

# Journal of Biosciences

Vol. 2, January-December 1980

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## **olution nuclear magnetic resonance studies of hen's egg yolk oproteins**

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ceived 4 December 1979

**ct.** High resolution nuclear magnetic resonance spectra of native or protease-treated egg yolk plasma (very low density lipoproteins) were taken either in water or deuterated. The protease-treated samples showed a sharpening of choline methyl proton signal of yolk lipid, indicating the hindrance of the choline head-group rotation by the phospholipid native very low density lipoproteins. With both native and the protease-treated egg yolk , elevated temperature increased the signal intensity and produced line-sharpening of

$\delta$  methyl protons and the  $\gamma\text{-CH}_2-\overset{\text{O}}{\underset{\text{C}}{\text{C}}}$  protons of the methylene group adjacent to the carboxyl group of esterified fatty acids, indicating prior restriction of mobility of these groups. Extracted lipids of egg yolk plasma containing traces of chloroform, methanol and water (keep the sample in one phase) also gave similar temperature dependence. Addition of  $\alpha$  the same sample and sonication resulted in the loss of temperature dependence. Frozen treated protease-treated egg yolk plasma also behaved in a similar manner. The absence of temperature dependence in these latter two samples is believed to be due to formation of s of phospholipids following phase separation of triglycerides and phospholipids. The support a model in which the lipoprotein particles of the egg yolk plasma have a lipid-core re containing triglycerides in the centre with a monomolecular layer of lecithin at the , the polar heads of which are surrounded by proteins.

**ords.** Nuclear magnetic resonance; egg yolk plasma; low density lipoproteins; phospho-

1

olk plasma very low density lipoprotein (VLDL) contains 13% protein, id has a density 0.98 g/ml (Cook and Martin, 1969; Evans *et al.*, 1973; ; Raju and Mahadevan, 1974). A lipid-core structure has been proposed , 1975; Eswaran and Mahadevan, 1972; Kamat *et al.*, 1972).

er report (Eswaran and Mahadevan, 1972) on the high resolution NMR e lipoprotein of egg yolk plasma, line broadening of the choline methyl l obtained was suggested to be due to a close association of the polar

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sed: Low density lipoprotein (VLDL); Nuclear magnetic resonance (NMR); proton mag- (PMR).

head of lecithin with polar areas on the apolipoprotein. A similar line broadening of the choline methyl proton signal in hen's egg yolk very low density lipoproteins (VLDL) was observed by Kamat *et al.* (1972) who suggested that this may be due to the interaction of the choline methyl group with the polar head of phosphatidyl ethanolamine, with triglycerides at an interface, or with the protein or polypeptide matrix. In contrast, the NMR signal obtained from the choline methyl protons in cholesterol ester-rich low and high density lipoproteins of human serum (Steim *et al.*, 1968; Chapman *et al.*, 1969 a, b; Leslie *et al.*, 1969) were pronounced and sharp, suggesting a possible difference in the location of the choline group in egg yolk VLDL and in human serum low density lipoprotein.

In this paper we present the proton magnetic resonance (PMR) spectra of the lipoproteins of egg yolk plasma before and after removal of the apolipoproteins by exhaustive treatment with a bacterial protease. The features of the spectra are compared with the PMR spectra of solvent-extracted total egg yolk plasma lipids either in single phase or following sonication in water. With all these samples the temperature dependence of the PMR spectra has also been recorded. The results are discussed in terms of the structural and motional properties of the components of VLDL.

### Materials and methods

One-day old, unfertilised White Leghorn eggs were procured from the poultry development section of the University of Agricultural Sciences, Hebbal, Bangalore. Granule-free egg yolk plasma or very low density lipoprotein (VLDL) was obtained by the procedure of Raju and Mahadevan (1974).

#### Protease treatment

Protein-depleted VLDL were prepared by exhaustive proteolysis of VLDL or egg yolk plasma with a protease (Ramakrishna Rao *et al.*, 1978). About 3 ml of egg yolk plasma or 4 ml of VLDL (180 mg of protein by Folin's method) in 0.05 M Tris-HCl buffer, pH 7.5, was treated with 18 mg of protease (ex. *Streptomyces griseus* Type VI, Sigma Chemicals, St. Louis, MO, USA) dissolved in 100 µl of the same buffer. The solution was taken in a dialysis bag with no air bubble in the tubing so as to keep volume changes to a minimum. The material was dialysed, with magnetic stirring, against three changes of 1 litre of 0.05M Tris-HCl buffer, pH 7.5, at 25-28°C for 48 h in order to remove all dialysable products of proteins digestion as and when formed. A few drops of toluene were added to the dialysing buffer to prevent microbial growth. After 48 h of proteolysis, the material was dialysed D<sub>2</sub>O (99.4-99.7%) containing 0.05M NaCl for a further 12 h, with 3 changes. A sample of egg yolk plasma with no protease addition was treated in exactly the same manner to serve as control.

A part of the control and protease-treated samples were analysed for protein content. The protease-treated egg yolk plasma or VLDL contained about 3% and 2% of the original protein content respectively. The product following protease treatment was therefore essentially the lipid-core or VLDL. Autodigestion and dialysis eventually removed most of the protease protein.

#### Protein estimation

Protein in control egg yolk plasma or VLDL was determined by the method of Lowry *et al.* (1951). However, in protease-treated samples, the small amounts of residual proteins in the presence of large amounts of lipids was estimated by a slight

modification of Lowry's procedure. Forty five minutes after the addition of the Folin's reagent, 1 ml of  $\text{CHCl}_3$  was added to 2.2 ml of the estimation mixture and mixed well using a vortex mixer. The emulsion formed was clarified by centrifugation for 10 min and the colour intensity of the top clear aqueous layer was measured colourimetrically.

#### *Induction of gelation by storing frozen samples*

This was achieved by placing control or protease-treated egg yolk plasma or VLDL samples directly in NMR tubes and storing at  $-20^\circ\text{C}$  for 6 days prior to thawing. NMR spectra of these gelled samples were recorded directly.

#### *Total lipids*

*Extraction of total lipids:* The total egg yolk plasma lipids were extracted from lyophilised egg yolk plasma by successive extraction with  $\text{CHCl}_3:\text{MeOH}$  (2:1) solvent mixture. The solvents were removed by flash-evaporation at  $30^\circ\text{C}$ .

*Total lipids in a single clear phase:* A clear deep yellow transparent oil was obtained by flash evaporation (when trace amounts of water, methanol and chloroform were present in the mixture). The NMR spectrum of this sample was recorded. Complete drying to remove all traces of solvents led to phase separation since the phospholipids are not soluble in the triglycerides present (Schneider *et al.*, 1968).

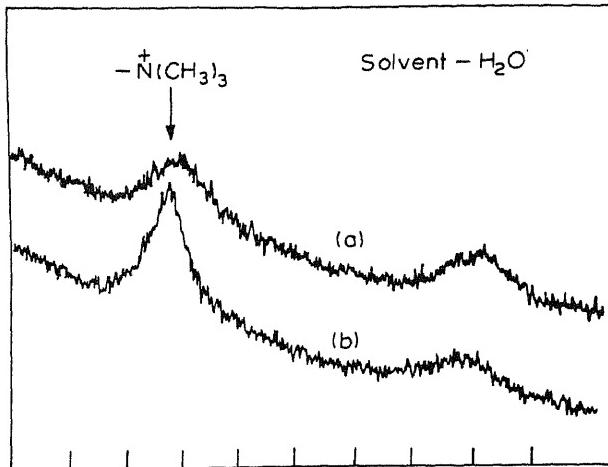
*Total lipids in water dispersion:* A part of the clear yellow single-phase oil of the total lipids was dispersed in water to give an emulsion, with a lipid concentration approximately that found in yolk plasma. The emulsion was sonicated for 20 min in a Branson sonifier, Model S-75 with cooling to prevent excess heating of sample during sonification. The resulting sonified emulsion was opaque cream coloured and viscous in appearance.

#### *PMR spectra*

PMR spectra were recorded on a Varian HA-100 NMR spectrometer at a temperature of  $31^\circ\text{C}$ . The spectrometer was usually on the water signal (ca.  $5.2 \tau$ ). All signals were recorded with trimethylsilane as internal reference. The temperature of the sample was varied using Varian temperature accessory and was measured to an accuracy of  $1^\circ\text{C}$ . Line widths were measured at half the signal intensity in Hz. The Varian C-1024 time-averaging computer was used to record the spectra of signals whenever necessary.

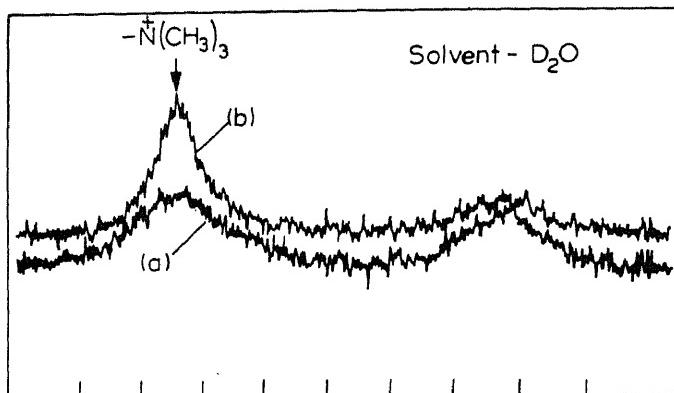
#### **Results**

Figure 1 and 2 show the PMR spectra of choline methyl proton signal of egg yolk plasma and protease-treated plasma in 0.05M NaCl in  $\text{H}_2\text{O}$  and  $\text{D}_2\text{O}$  respectively. Table 1 gives the average line width for the choline methyl proton signal following the treatments. The line width of the average signals of 11.1 Hz in the control egg yolk plasma (or VLDL) sample in water is reduced to 7.7 Hz upon protease treatment. In  $\text{D}_2\text{O}$ , the line width is higher (14.4 Hz) in the native sample and reduces to 9 Hz upon protease treatment. The  $\text{D}_2\text{O}$  effect is reversible and replacement of  $\text{D}_2\text{O}$  by  $\text{H}_2\text{O}$  brings back the egg yolk plasma choline methyl proton signal to 11.5 Hz.



**Figure 1.** PMR signal of choline methyl proton of egg yolk plasma (a) and protease-treated egg yolk plasma (b) in  $\text{H}_2\text{O}$ .

Temp.  $31^\circ\text{C}$ ; sample in 0.05M NaCl in  $\text{H}_2\text{O}$ .



**Figure 2.** PMR signal of choline methyl proton of egg yolk plasma (a) and protease-treated egg yolk plasma (b) in  $\text{D}_2\text{O}$ .

Temp.  $31^\circ\text{C}$ ; sample 0.05M NaCl in  $\text{D}_2\text{O}$ .

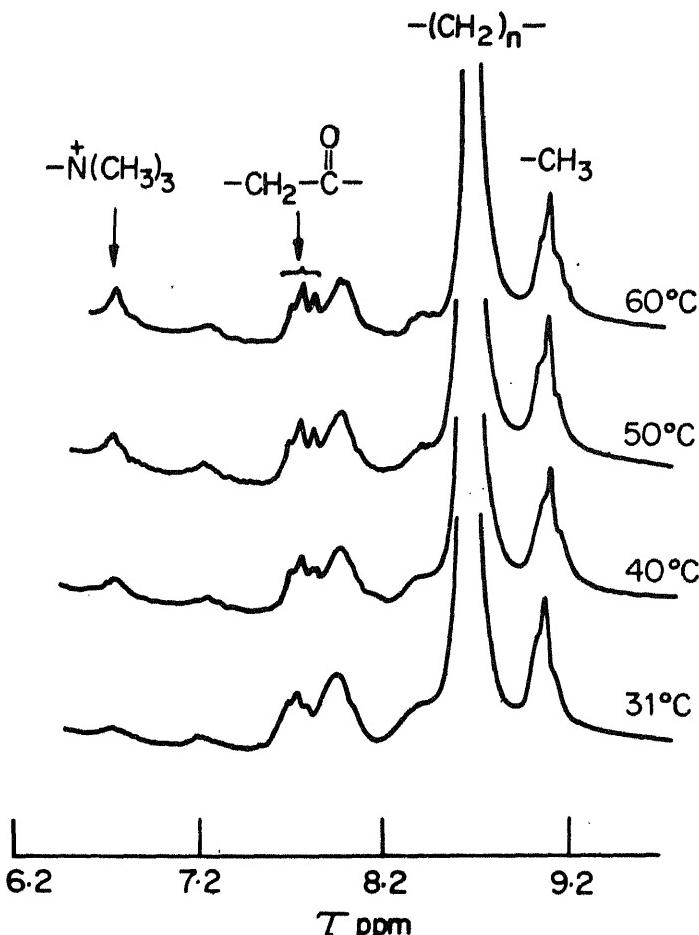
**Table 1.** Line width of choline methyl proton signal of egg yolk plasma and protease-treated egg yolk plasma.

Sample	Average line width (Hz)	
	in $\text{H}_2\text{O}$	in $\text{D}_2\text{O}$
Egg yolk plasma*	11.1	14.4
Egg yolk plasma (protease-treated*)	7.7	9.0
Egg yolk plasma in $\text{D}_2\text{O}$ and back in $\text{H}_2\text{O}$	11.5	—

\* Average of 3 experiments; temperature of measurement =  $31^\circ\text{C}$ .

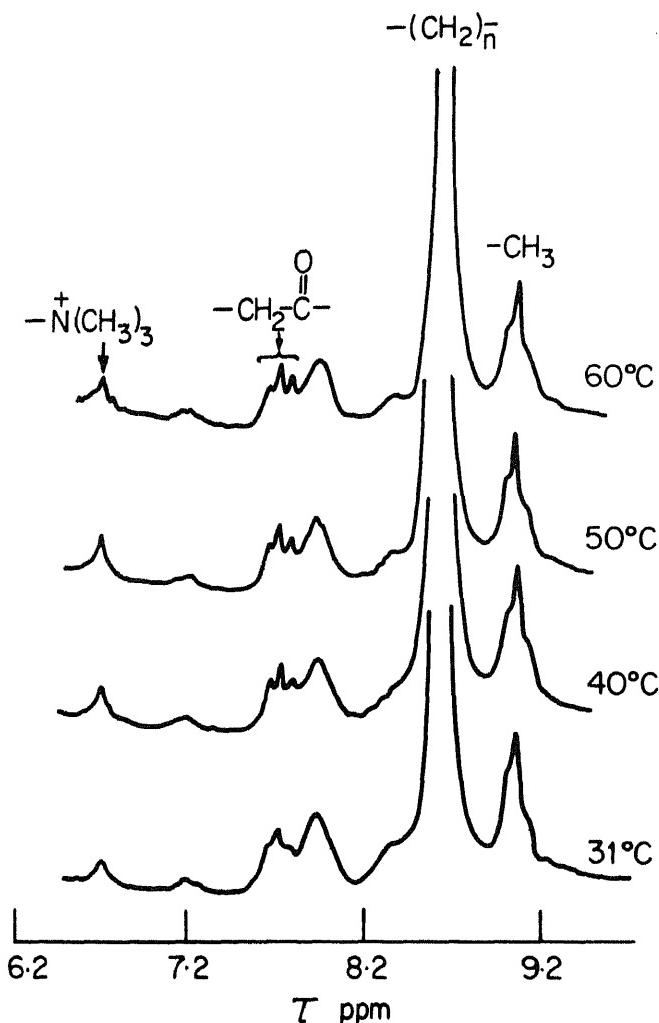
Figures 3 and 4 show the temperature dependence of the PMR spectra of egg yolk plasma and of plasma treated with protease in 0.05M NaCl in  $\text{H}_2\text{O}$ . At the higher temperatures, the choline methyl proton signals became sharper both in egg yolk plasma and plasma treated with protease. In addition, the triplet signal at 7.7 ppm,

and assigned to  $-\text{CH}_2-\overset{\text{O}}{\underset{\parallel}{\text{C}}}-$  protons of methylene group adjacent to the carboxyl group of the esterified fatty acids of triglycerides and phospholipids, becomes very prominent at 60°C with measurable separation, when compared to 30°C where it is a broad single peak. Thus increase in temperature increases the signal intensity and brings about line sharpening of choline methyl protons and gives rise to sharp signals with fine structure of the  $-\text{CH}_2-\overset{\text{O}}{\underset{\parallel}{\text{C}}}-$  protons. The methylene ( $-\text{CH}_2-$ )<sub>n</sub> and methyl ( $-\text{CH}_3$ ) groups of the esterified fatty acids are relatively unaffected by increased temperature. The line widths as a function of temperature change for the choline methyl and  $-\text{CH}_2-\overset{\text{O}}{\underset{\parallel}{\text{C}}}-$  protons signals are given in table 2. There is a pronounced and progressive reduction in the line widths of  $-\text{CH}_2-\overset{\text{O}}{\underset{\parallel}{\text{C}}}-$  proton signals



**Figure 3.** Temperature dependence of PMR spectra of egg yolk plasma in  $\text{H}_2\text{O}$ . Samples in 0.05M NaCl in  $\text{H}_2\text{O}$ .

from 11.5-12 Hz at 31°C to 6-7 Hz at 60°C in both egg yolk plasma and protease-treated egg yolk plasma samples. However protease treatment *per se* does not appear to affect the line width of these signals as it affects the line width of the choline methyl proton signals.



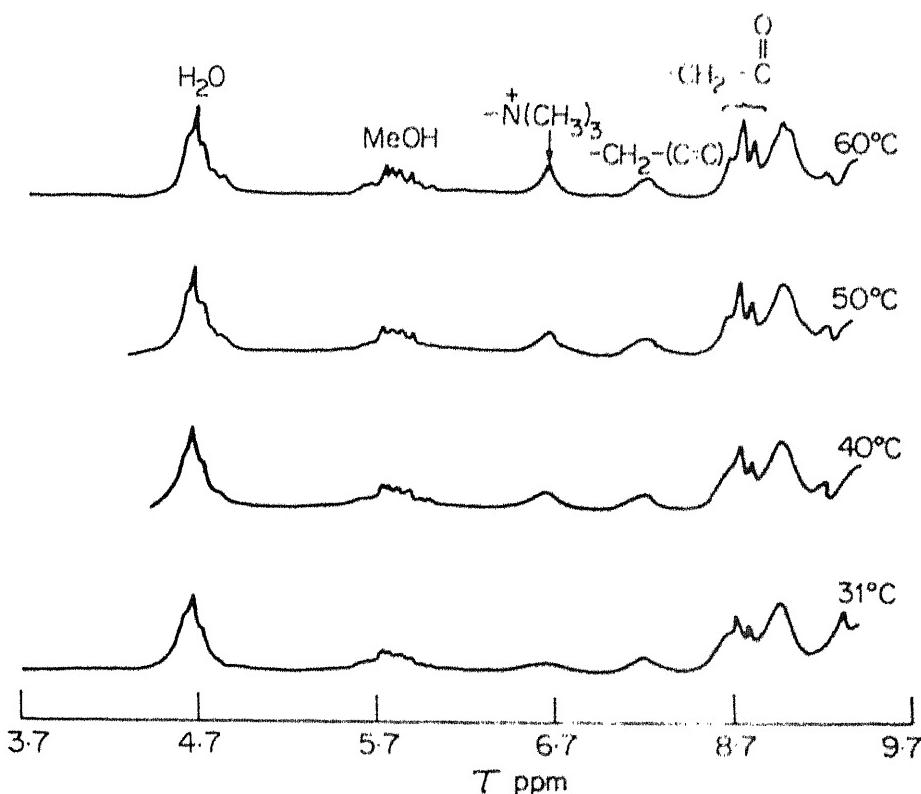
**Figure 4.** Temperature dependence of PMR spectra of protease-treated egg yolk plasma in  $\text{H}_2\text{O}$ . Sample in 0.05M NaCl in  $\text{H}_2\text{O}$ .

The PMR spectra and the temperature dependence of total extracted egg yolk sma lipids is given in figure 5. The clear yellow sample in single phase contained 25% of water, methanol and chloroform as already described (cf. Experimental). The spectrometer was locked on the large ( $-\text{CH}_2-$ )<sub>n</sub> signal which enable the detection of  $\text{H}_2\text{O}$ , methanol and  $\text{CHCl}_3$  proton signals. It is clearly seen that the broad choline signal is sharpened with an increase in temperature upto 60°C. However, these signals are still somewhat broad compared to the egg yolk plasma or the

**Table 2.** Temperature dependence of line width of choline methyl and  $-\text{CH}_2-\overset{\text{O}}{\underset{\text{C}}{\text{C}}}-$  proton signals (in  $\text{H}_2\text{O}$ ).

Temp. °C	Egg yolk plasma		Egg yolk plasma protease treated	
	$\text{N}^+(\text{CH}_3)_3$	$\text{CH}_2-\overset{\text{O}}{\underset{\text{C}}{\text{C}}}-$ triplet	$\text{N}^+(\text{CH}_3)_3$	$\text{CH}_2-\overset{\text{O}}{\underset{\text{C}}{\text{C}}}-$ triplet
	Hz	Hz	Hz	Hz
31	11.0	12, 12*	8.5	12, 11.5*
40	10.0	10, 9.5*	8.0	8, 9.5*
50	7.0	6.5, 7, 7.5	6.5	7, 6.5, 6.5
60			6.1	6.5, 6, 7

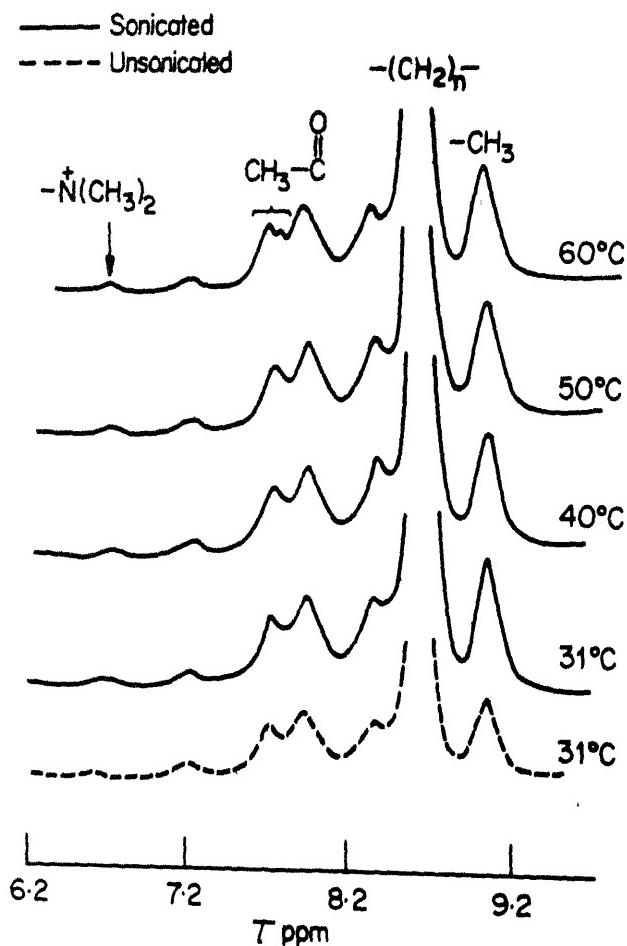
\* Initial triplet signal present as a shoulder and was not measurable.



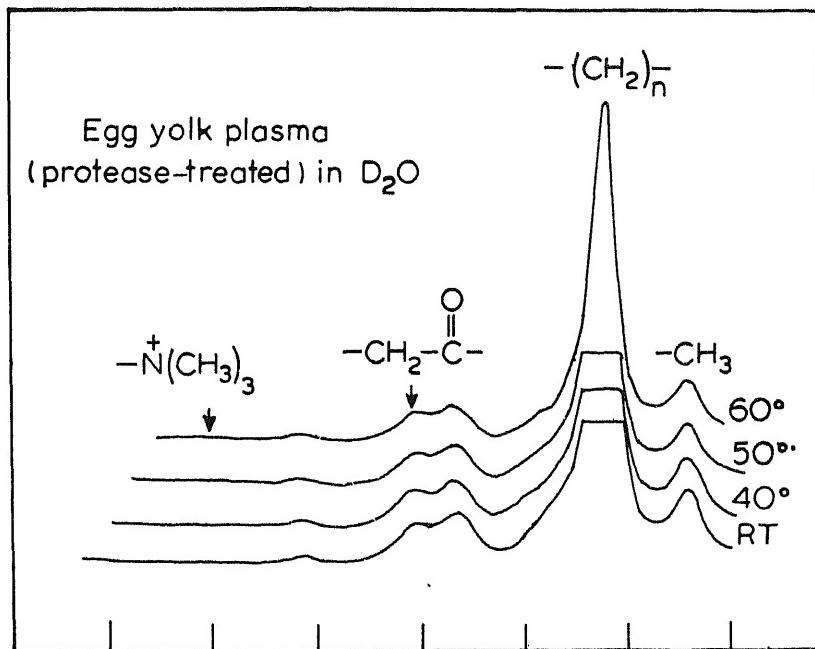
**Figure 5.** Temperature dependence of PMR spectra of total extracted lipids of egg yolk plasma in single phase.

protease-treated plasma choline methyl proton signals seen in figures 3 and 4. The  $-\text{CH}_2-\overset{\text{O}}{\underset{\text{H}}{\text{C}}}-$  proton signal is likewise sharpened with temperature.

The PMR spectra at various temperature ( $31^\circ\text{C}$  to  $60^\circ\text{C}$ ) of the total lipids of egg yolk plasma were dispersed in water by sonication is reproduced in figure 6. In this the choline methyl proton signals remained broad and unchanged upto  $60^\circ\text{C}$ . The  $-\text{CH}_2-\overset{\text{O}}{\underset{\text{H}}{\text{C}}}-$  proton signals were likewise unaffected and no triplet signals are seen at the elevated temperatures. However the fatty acid methyl ( $-\text{CH}_3$ ) signal appears to sharpen slightly.

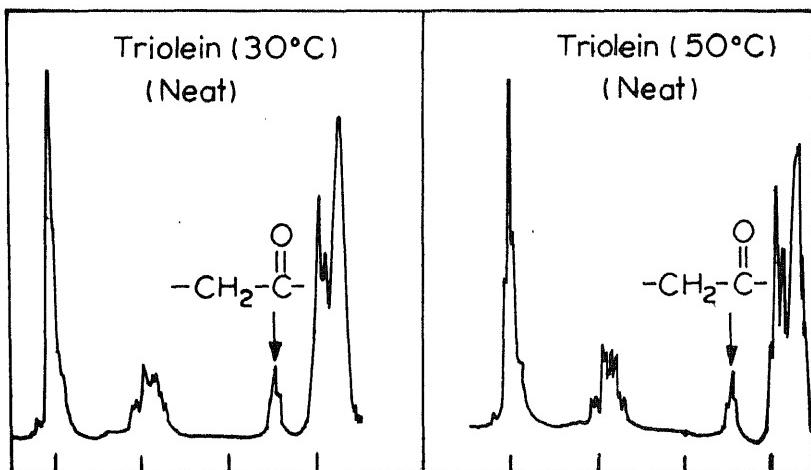


**Figure 6.** Temperature dependence of PMR spectra of total lipids of egg yolk plasma dispersed in water.



**Figure 7.** Temperature dependence of PMR spectra of protease-treated egg yolk plasma stored frozen and thawed.

Figure 7 shows the temperature dependence of PMR of protease-treated egg yolk plasma (in  $D_2O$ ) stored frozen and thawed. Upon storage in the frozen state and thawing, the sample which was initially a clear yellow fluid becomes an opaque yellow gel, obviously caused by phase separation of the triglycerides and phospholipids. In the PMR spectra of such a sample, the choline methyl proton signal is almost non-existent and the signals due to  $-CH_2-C(=O)-$ ,  $(-CH_2)_n$  and  $-CH_3$



**Figure 8.** Temperature dependence of PMR spectra of triolein.

groups are quite broad. The spectrum is very similar to that of the total egg yolk plasma lipids dispersed in water (figure 6). Increase in temperature had hardly any effect on any region of the spectrum including choline methyl and  $-\text{CH}_2-\overset{\text{O}}{\underset{\parallel}{\text{C}}}-$  proton regions.

The PMR spectra of triolein, which was taken as a model for an unsaturated yolk triglyceride, was recorded neat at 31°C and 50°C and is shown in figure 8. The  $-\text{CH}_2-\overset{\text{O}}{\underset{\parallel}{\text{C}}}-$  signals shows a triplet nature at 31°C which is unchanged at 50°C. However all other signals sharpened on going to the elevated temperature.

### Discussions

The decrease in the line width of the choline methyl  $[-\text{N}^+(\text{CH}_3)_3]$  protons in the protease-treated samples, in both H<sub>2</sub>O and D<sub>2</sub>O, clearly support the hypothesis that line broadening is due to the hindrance of choline head group rotation by polypeptide in the native lipoprotein. The temperature-dependent enhancement of signal intensity

of both choline methyl and  $-\text{CH}_2-\overset{\text{O}}{\underset{\parallel}{\text{C}}}-$  group protons in native as well as protease-treated lipoprotein indicate that temperature preferentially increases the mobility of these groups when compared to the methylene  $(-\text{CH}_2-)_n$  and methyl groups. Similar effects were observed with the total extracted lipids in single phase. However, in the spectra of the extracted lipids dispersed in water the choline methyl and

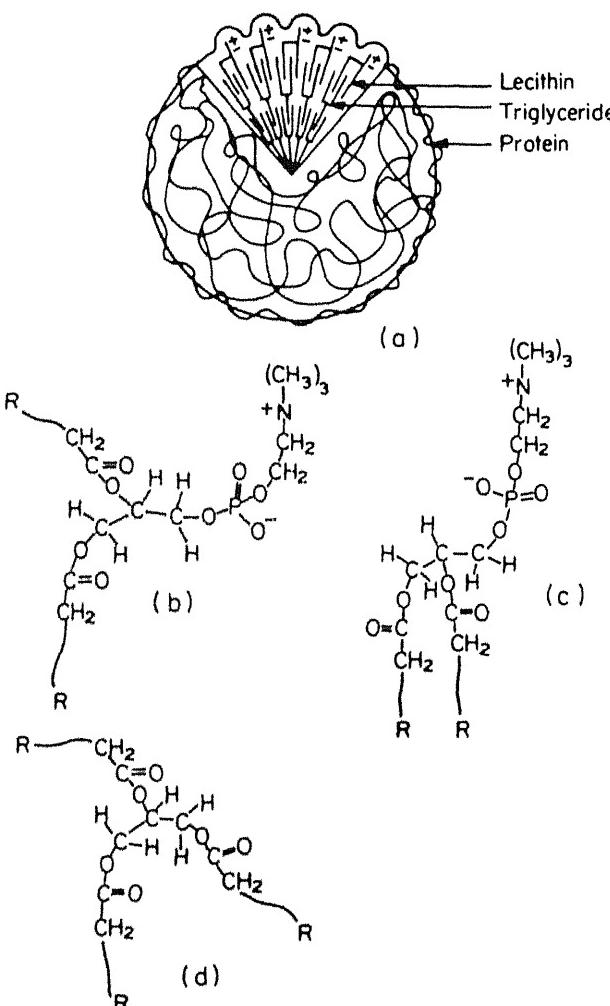
$-\text{CH}_2-\overset{\text{O}}{\underset{\parallel}{\text{C}}}-$  proton signals are hardly affected by an increase in temperature probably indicating a different state of aggregation of these lipids compared with that in native or protease-treated egg yolk plasma.

A temperature-dependence study on a model triglyceride, triolein, indicated no sharpening of the triplet signal of  $-\text{CH}_2-\overset{\text{O}}{\underset{\parallel}{\text{C}}}-$  at the elevated temperature though all other signals sharpened. These protons are apparently quite unhindered for rotation even at the lower temperature and therefore temperature-insensitive. The results therefore suggest that the temperature-dependent  $-\text{CH}_2-\overset{\text{O}}{\underset{\parallel}{\text{C}}}-$  proton triplet signals in egg yolk plasma or protease-treated plasma may arise from the esterified fatty acids of phospholipids.

All these results support a model as shown in figure 9a and figure 10a where the lipoprotein particles of egg yolk plasma is shown as a lipid core structure containing triglycerides in the centre with a monomolecular layer of phospholipids on the surface, the polar head groups of which are surrounded by proteins. Such a structure has been proposed by Kamat *et al.* (1972). In this structure the motion of the choline

methyl and  $-\text{CH}_2-\overset{\text{O}}{\underset{\parallel}{\text{C}}}-$  protons are restricted. In the case of choline this may be due to the interaction of the polar groups (phosphate and  $\text{N}^+(\text{CH}_3)_3$ ) with polar groups of the protein. Protease treatment removes the proteins which, at least

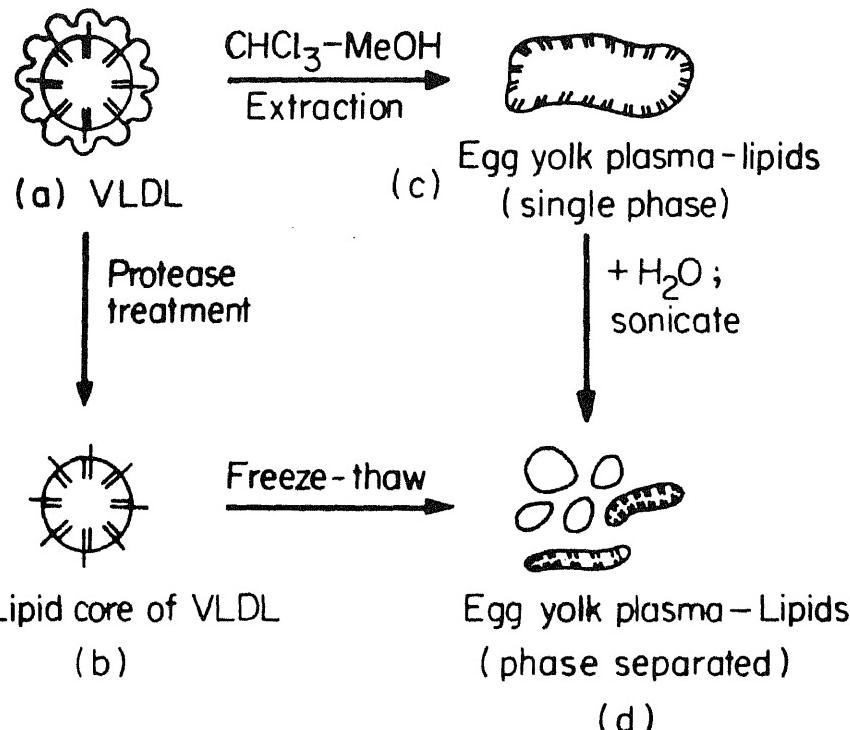
partly, releases the choline head group from this interaction. In the case of  $-\text{CH}_2-\overset{\text{O}}{\underset{\parallel}{\text{C}}}-$  group protons, the restriction may be due to the necessary closer packing of both the fatty acid chains of phospholipids into the triglyceride core, thereby hindering the freedom of motion. Figure 9b and 9c show lecithin molecules with fatty acids chains 'close' packed with less freedom of movement or 'open' packed with greater



**Figure 9.** (a) Lipid-core model of VLDL. Partly cut away to show packing of lecithin and triglyceride. Polar head groups of lecithin in close association with protein on the surface of the lipid core. (b) Lecithin molecule with 'open' arrangement of fatty acid chains. (c) Lecithin molecule with 'close' arrangement of fatty acid chains. (d) Triglyceride molecule with 'open' arrangement of fatty acid chains.

rotational freedom. Triglycerides in bulk phase have no restriction of motion of the three fatty chains (figure 9d). On protease treatment, the lipid cores of these lipoprotein particles are released into the medium wherein the triglycerides and phospholipids apparently maintain their initial packing arrangement, i.e. triglyceride cores surrounded by a phospholipid monolayer (figure 10b). At this stage, a certain amount of coalition of the lipid cores of these lipoproteins probably occurs, since the volume of these lipid cores is somewhat larger than that of VLDL particles as determined by Sepharose 4B gel filtration (unpublished results). Since the protein restriction has been removed, the signals from choline methyl protons appears

sharpened. However, the motion of  $-\text{CH}_2-\text{C}-$  grouping of the phospholipid is still restricted, which is reduced at higher temperature as with the whole lipoprotein



**Figure 10.** Schematic representation of changes in the packing of lipid components of egg yolk plasma following various treatments. (a) Intact VLDL particle with lipid core and protein coat surface. Phospholipid molecules arranged in monolayer over a bulk triglyceride core. (b) Protease-treated VLDL particle with the protein coat removed. Arrangements of lipids as in (a). (c) Total extracted egg yolk plasma lipids in single phase. Phospholipids shown as monolayer dispersing triglycerides. (d) Frozen and thawed particle showing phase separation of triglycerides (open figure) and phospholipids in bilayer. Same phase separation is obtained by adding water to extracted egg yolk plasma lipids (c).

particle. In the total extracted egg yolk plasma lipid sample containing traces of water,  $\text{CHCl}_3$  and methanol (figure 10c), where there is no phase separation of triglycerides and phospholipids, the phospholipids probably occur in the monolayer form keeping the triglycerides in some sort of a dispersed state. Such a sample shows a temperature dependence similar to egg yolk plasma protease-treated plasma. Protease-treated egg yolk plasma sample, stored frozen and thawed, becomes opaque probably due to phase separation of the triglycerides and phospholipids (figure 10d). Similar phase separation apparently occurs when water is added to extracted total egg yolk plasma lipids (figure 10d). Both these samples showed no temperature

dependence of  $-\text{N}^+(\text{CH}_3)_3$  and  $-\text{CH}_2-\overset{\text{O}}{\underset{\text{H}}{\text{C}}}-$  proton signals as explained earlier. Phase separation in the frozen and thawed, protease-treated egg yolk plasma sample was also indicated by low angle x-ray scattering which was not obtained with the unfrozen sample (Mahadevan and Chapman, unpublished results).

#### Acknowledgement

Research financed by a grant to one of us (SM) from the U S Department of Agriculture under PL-480 project No. FG-In-477.

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## **Regulation of rat liver NADP<sup>+</sup>-isocitrate dehydrogenase during aging**

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MS received 5 January 1980; revised 1 March 1980

**Abstract.** In an attempt to understand the mechanism of aging in relation to the differences in enzyme regulation, the induction and kinetic properties of NADP<sup>+</sup>-isocitrate dehydrogenase of the liver of immature (6 weeks), mature (13 weeks), adult (33 weeks) and old (85 weeks) female rats were studied. The specific activity of the cytoplasmic and mitochondrial NADP<sup>+</sup>-isocitrate dehydrogenase increased up to the adult age (33 weeks) and decreased in the old rats (85 weeks). Ovariectomy decreased and estradiol administration induced activity of both the mitochondrial and cytoplasmic enzyme in the liver of immature, mature and adult rats but had no significant effect in old rats. However, the activity of mitochondrial NADP<sup>+</sup>-isocitrate dehydrogenase decreased and cytoplasmic NADP<sup>+</sup>-isocitrate dehydrogenase increased following ovariectomy in old rats (85 weeks). Hormone-mediated induction of enzyme activity was actinomycin D sensitive. The K<sub>m</sub> for isocitrate and NADP, K<sub>i</sub> value for oxalomalate, heat stability and electrophoretic mobility of the purified enzyme from the cytosol fraction of the liver of immature and old rats were similar. It can be concluded that the enzyme does not change structurally with age.

**Keywords.** Isocitrate dehydrogenase; induction; purification; electrophoretic mobility; temperature sensitivity.

### **Introduction**

The activities of several enzymes either decrease, or increase as a function of the age of the organism (Kanungo, 1970). A possible cause for such changes may be related to the template activity of the corresponding gene(s). We have studied two isoenzymes of NADP<sup>+</sup>-linked isocitrate dehydrogenase (EC 1.1.1.42), from the cytoplasm and mitochondria as markers to obtain some insight into this problem.

Although alterations in the isoenzyme pattern of lactate dehydrogenase (EC 1.1.1.27) of various tissues as a function of age of the rats have been reported (Singh and Kanungo, 1968), the induction of the isoenzymes of NADP<sup>+</sup>-isocitrate dehydrogenase has not been studied. Both cytosolic and mitochondrial NADP<sup>+</sup>-isocitrate dehydrogenase differ in their electrophoretic mobility and immunological pattern (Lowenstein and Smith, 1962), suggesting that their synthesis may be governed by two separate genes. The cytoplasmic enzyme supplies NADP<sup>+</sup> for reductive biosynthesis (Lowenstein, 1961), whereas mitochondrial enzyme is involved in the production of energy (Stein *et al.*, 1967). It has been reported that sex steroids modulate this enzyme in the brain and pituitary of rats (Luine, *et al.*, 1974, 1975). It was of interest to investigate the changes that occur in the isoenzymes of NADP<sup>+</sup>-isocitrate dehydrogenase of the liver during aging of the rats. These include alterations in the levels of the isoenzyme and regulation.

## Materials and methods

### Materials

Albino rats of Wistar strain (*Rattus norvegicus albinus*) were used. They were maintained at  $24 \pm 2^\circ\text{C}$  with 12 h light period followed by 12 h dark period. The rats were fed on freshly prepared diet containing flour and vitaminised milk-powder in the ratio of 4:1 in water with added table salt. The diets were supplemented with gram (*Cicer arietinum*) on alternate days. Tap water was supplied *ad libitum*. Female rats of four age groups, representing immature (6), mature (13), adult (33) and old (85 weeks) were used in this study. All chemicals used were of the analytical grade. All the biochemicals were purchased from Sigma Chemical Co., St. Louis, Missouri, USA.

### Assay of isoenzymes

The rats were killed by cervical dislocation, the liver was excised, chilled in ice-cold 0.9% NaCl. A 10% homogenate was prepared in 0.25 M sucrose containing 0.1 mM EDTA and centrifuged at 700 g for 10 min in a high-speed IEC (PR-6 model) refrigerated centrifuge. The supernatant was again centrifuged at 14,000 g for 30 min. The supernatant obtained was used for the assay of cytoplasmic NADP<sup>+</sup>-isocitrate dehydrogenase. The pellets were suspended in 0.25 M sucrose containing 0.1 mM EDTA. To solubilise the enzyme, an equal volume of phosphate buffer (0.1 M, pH 7.4) containing 10% glycerol and 0.5% Triton X-100 was added to the mitochondrial suspension which was then homogenised and centrifuged. This was used for the assay of mitochondrial enzyme by monitoring the NADPH produced. Both the isoenzymes were assayed in an Unicam SP-500 spectrophotometer (Watanabe *et al.*, 1974). One unit of enzyme is defined as that amount which forms 1  $\mu\text{mol}$  NADPH/min at  $25^\circ\text{C}$ . The protein content of the fractions was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. The data were statistically analysed for Student's 't' test according to Garret (1966).

### Effect of ovariectomy and estradiol on isocitrate dehydrogenase isoenzymes

The rats of all the ages were divided into four groups, each group comprised of 4-5 rats. Rats in group I were injected with 1.0 ml of 0.9% NaCl intraperitoneally. Rats in groups II, III and IV were bilaterally ovariectomised and maintained for 21 days. On the 22nd day, rats in group II received 1.0 ml 0.9% NaCl injection and served as ovariectomised controls. Rats in group III were administered estradiol (20  $\mu\text{g}/100\text{ g}$  body weight in 1.0 ml of 0.9% NaCl) intraperitoneally. Rats in group IV were injected actinomycin D (10  $\mu\text{g}/100\text{ g}$  body weight in 1.0 ml of 0.9% NaCl) 1 h prior to the hormone administration. All the injections were given intraperitoneally at 2.00 p.m. and the rats were killed at 6.00 p.m. on the same day.

### Purification of NADP<sup>+</sup>-isocitrate dehydrogenase

The NADP<sup>+</sup>-isocitrate dehydrogenase was purified from the cytosol fraction of the liver of immature (6 weeks) and old (85 weeks) rats by the procedure described by Islam (1972) with certain modifications. All the operations were carried out at  $2^\circ\text{C}$  unless otherwise indicated. The livers from 5-6 rats were pooled for each age group.

## Results

### Levels of isoenzyme

The levels of both cytoplasmic NADP<sup>+</sup>-isocitrate dehydrogenase and mitochondrial NADP<sup>+</sup>-isocitrate dehydrogenase increased during active growth phase and adulthood (table 1). Thereafter, the activity decreased significantly in old rats of 85 weeks.

### Effect of ovariectomy and estradiol on isoenzymes

Table 1 shows that the levels of both the isoenzymes of the liver of rats in the different age groups decrease significantly following ovariectomy, except that the cytoplasmic enzyme of old rats (85 weeks) increased significantly. Estradiol administration induced both the isoenzymes in all the age groups. However, the mitochondrial enzyme of old rats (85 weeks) did not show any significant change on estradiol treatment. The degree of response was greater in 6 and 13 weeks as compared to that of 33- and 85-weeks old rats. Actinomycin D injection to ovariectomised and estradiol treated rats restored the enzyme level to that in the normal ovariectomised control rats (table 1).

### Studies with the purified enzyme

Table 2 gives the protocol for the purification of NADP-isocitrate dehydrogenase from the liver cytosol of immature (6) and old (85 weeks) normal female rats, respectively for the enzyme from 6 and 85 week-old rats. Figure 1 gives the elution pattern of enzymes from livers of rats of two ages from DEAE-cellulose columns. The enzyme from both the ages were eluted at the same concentration of KCl. The apparent  $K_m$  values for isocitrate and NADP<sup>+</sup> are the same for the enzyme from both, 6 and 85 week old rats (table 3). The  $K_i$  value for oxalomalate were 8.5  $\mu\text{M}$  and 9.0  $\mu\text{M}$  for the immature and the old rats respectively (table 3). The activity of purified enzyme from the two age groups of rats at different temperatures (figure 2) were similar.

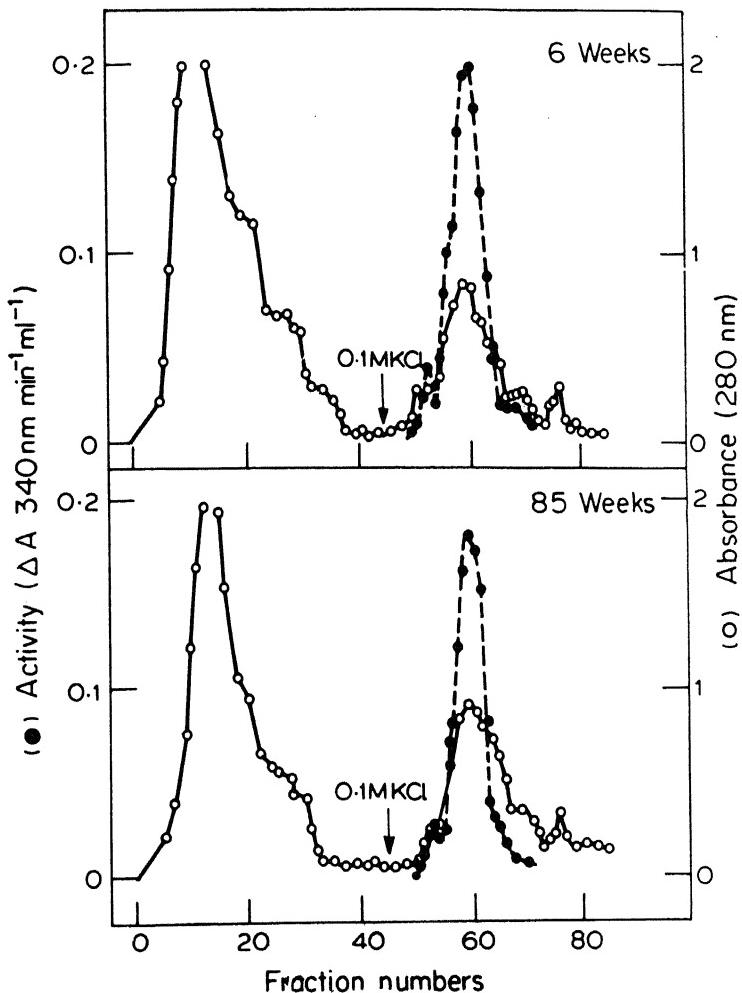
The enzyme from both immature and old rats had identical mobility when subjected to polyacrylamide gel electrophoresis at 4°C in 0.01M citrate phosphate buffer pH 1.0, containing 10% glycerol and 0.1 mM EDTA. A constant current of 4 mA/gel was applied for 3 h. One of the gels was stained for protein with 0.25% coomassie brilliant blue R (prepared in 10% trichloroacetic acid) after fixing it with 12.5% trichloroacetic acid for 1 h. For specific staining of NADP<sup>+</sup>-isocitrate dehydrogenase activity, the gels were incubated in the staining mixture for 30 min at 37°C. The Staining mixture consisted of 0.1 M Tris/HCl pH 7.4, 8.0 mM isocitrate, 4.0 mM MgCl<sub>2</sub>, 0.26 mM NADP, 0.25 mM nitroblue tetrazolium and 0.18 mM phenazine methosulphate according to the method of Henderson (1965).

**Table 1.** Effects of ovariectomy, estradiol and actinomycin D on the activity levels of NADP-isocitrate dehydrogenase isoenzymes in the liver of female rats of various ages.

Treatments	Specific activity of NADP <sup>+</sup> -isocitrate dehydrogenase isoenzymes							
	Mitochondrial enzyme			Cytoplasmic enzyme				
	6	13	33	85	6	13	33	85
Normal	129.03±1.41(5)	163.02±2.86(6)	130.85±3.48(5)	112.48±0.86(5)	297.58±3.85(5)	335.97±6.55(6)	317.50±3.54(5)	253.10±1.98(5)
Ovariectomized	74.02±8.56(5) <i>P</i> <0.01	101.18±0.75(6) <i>P</i> <0.001	86.68±0.43(5) <i>P</i> <0.001	102.16±1.47(4) <i>P</i> <0.01	243.10±2.90 <i>P</i> <0.001	264.10±4.01(6) <i>P</i> <0.001	264.72±1.28(5) <i>P</i> <0.001	279.03±6.78(4) <i>P</i> <0.01
Ovariectomized+estradiol	131.98±2.87(5) <i>P</i> <0.01	152.68±2.76(5) <i>P</i> <0.001	112.59±2.36(5) <i>P</i> <0.001	104.31±1.90(5) <i>P</i> : NS	317.99±0.77(5) <i>P</i> <0.001	353.18±1.96(5) <i>P</i> <0.001	301.55±5.62(5) <i>P</i> <0.01	297.13±2.93(5) <i>P</i> <0.05
Ovariectomized+actinomycin D+estradiol	88.10±7.00(4) <i>P</i> <0.01	84.10±1.27(5) <i>P</i> <0.001	103.29±2.06(4) <i>P</i> <0.02	96.30±1.10(5) <i>P</i> <0.01	203.32±3.47(4) <i>P</i> <0.001	265.74±4.17(5) <i>P</i> <0.001	243.72±6.68(4) <i>P</i> <0.01	237.61±4.36(5) <i>P</i> <0.001
					243.10±2.90(5)			

**Table 2.** Summary of partial purification of cytoplasmic NADP<sup>+</sup>-isocitrate dehydrogenase from liver of immature (6 weeks) and old (85 weeks) normal female rats

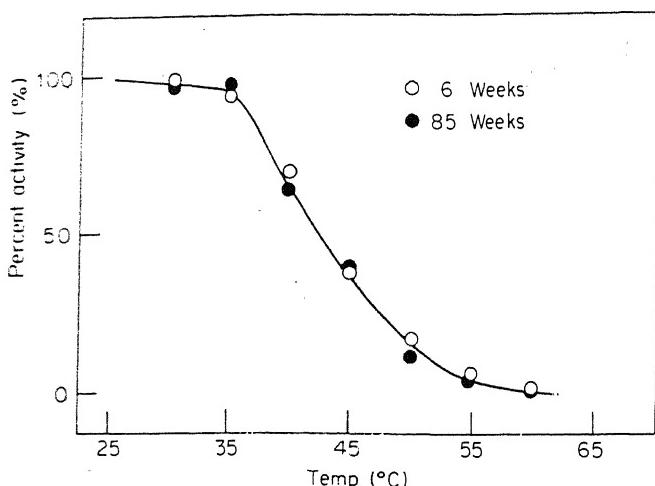
Age	Fractions	Volume (ml)	Total enzyme activity (units)	Total protein (mg)	Specific activity (units mg protein)	Fold purification	Yield (%)
6 weeks	Supernatant (40,000 g)	95	228.95	939.12	0.24	—	100
	First (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fraction (50-70%)	30	179.70	214.10	0.57	2.33	78.48
	Second (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fraction (40-60%)	20	154.20	167.92	0.91	3.76	67.35
	DEAE-cellulose chromatography fraction	20	76.28	17.89	4.26	17.49	33.31
	Third (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fraction (51-69%)	5	42.35	6.26	6.76	27.75	18.49
85 weeks	Supernatant (40,000 g)	90	234.16	985.45	0.23	—	100
	First (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fraction (50-70%)	32	189.45	348.94	0.54	2.28	80.89
	Second (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fraction (40-60%)	18	150.63	184.45	0.81	3.43	64.31
	DEAE-cellulose chromatography fraction	25	69.45	18.14	3.82	16.11	29.65
	Third (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fraction (51-69%)	6	38.02	6.56	5.79	24.39	16.23



**Figure 1.** Elution pattern of  $\text{NADP}^+$ -isocitrate dehydrogenase of liver cytosol of immature (6 weeks) and old (85 weeks) normal female rats on DEAE-Cellulose column.  
 (●—●) enzyme activity, (○—○) protein absorbancy at 280 nm.

**Table 3.**  $K_m$  and  $K_i$  of cytoplasmic- $\text{NADP}^+$ -isocitrate dehydrogenase of the liver of immature (6-weeks) and old (85-weeks) normal female rats

	6 weeks	85 weeks
$K_m$ for isocitrate	$6.00 \times 10^{-6}$ M	$6.40 \times 10^{-6}$ M
$K_m$ for $\text{NADP}^+$	$2.70 \times 10^{-5}$ M	$2.85 \times 10^{-5}$ M
$K_i$ for oxalomalate	$8.50 \times 10^{-5}$ M	$9.00 \times 10^{-5}$ M



**Figure 2.** Effect of temperature on the activity of cytoplasmic NADP<sup>+</sup>-isocitrate dehydrogenase of liver of immature (6 weeks) and old (85 weeks) normal female rats.

Appropriately diluted solutions of purified enzyme of immature and old rats were incubated for 15 min at a fixed temperature in a thermostatically controlled waterbath. The samples were then cooled quickly at 25°C and the enzyme activity was assayed.

## Discussion

The functional capacity of an organism deteriorates with increasing age (Shock, 1960). The decline in the functional or metabolic capacity of the organism during aging may be due to alterations in the activity and inducibility of certain important rate-limiting enzymes of various metabolic pathways (Finch *et al.*, 1969; Chainy and Kanungo, 1978). Such alterations may be attributed to the differential template activity of the corresponding gene(s) (Kanungo, 1970).

In the present study, the high levels of both the isoenzymes of NADP<sup>+</sup>-isocitrate dehydrogenase during active growth phase and adulthood may be correlated with the high degree of physiological activities of animal during this phase of life. The step in the Krebs cycle, catalysed by isocitrate dehydrogenase occupies a central position in the intermediary metabolism and links a variety of synthetic and energy yielding pathways (Srere and Bhaduri, 1962). Furthermore, the metabolic step controlled by this enzyme could be rate limiting (Kadenbach *et al.*, 1964). Therefore, the high level of the isoenzymes in the young animal and decrease in the activity in the old animal, observed in this study, supports the above hypothesis.

Pitot and Yatvin (1973) reported that the removal of the organ secreting a hormone from an animal causes a change in the levels of many enzymes in different tissues (Santti and Villee, 1971; Singhal, 1967; Gandhi and Kanungo, 1974; Giri and Singh, 1978a, b; Chainy and Kanungo, 1978). Our investigation indicates that the level of both the isoenzymes of NADP<sup>+</sup>-isocitrate dehydrogenase *in vivo* is regulated, at least in part, by sex steroids throughout the life span of the rat. It is noteworthy that the degree of response to ovariectomy or estradiol treatment is maximum at maturity and thereafter decreases in old rats. This finding confirms that estradiol not only maintains the secondary sexual characters for the attainment of puberty but also increases the overall physiological activities of the animal during this phase of life.

The decrease in the response in old age may be due to the loss of ovarian function. Furthermore the possible decrease of estradiol specific receptors in the liver of old rats may in turn decrease the responsiveness of its gene (Kanungo *et al.*, 1975).

The induction of an enzyme by a hormone may be due to, (i) stimulation of transcription or translation of the mRNA specific for that enzyme; or (ii) a decrease in the rate of degradation of the enzyme; or (iii) stimulation of the rate of synthesis of a modifier molecule which interacts with the enzyme and enhances its catalytic efficiency without any change in its net synthesis or degradation (Cox *et al.*, 1971). Our observations that the induction of the enzyme was inhibited by actinomycin D show that the effect may be due to stimulation of transcription of specific mRNA. The conclusion was in agreement with the finding of DeAsua *et al.*, (1968) that the induction of pyruvate kinase by estradiol in the uterus of ovariectomised rats was inhibited by actinomycin D.

Studies on the purified enzyme from the cytoplasmic fraction of the liver of 6- and 85-week old rats showed that the structure and net charge of the enzyme was independent of the age of the animal. In addition the K<sub>m</sub> and K<sub>i</sub> values for substrates and inhibitor were similar.

The observations that the kinetic properties of alanine amino transferase (Patnaik and Kanungo, 1976) and the kinetic and immunological properties of superoxide dismutase (Reiss and Gershon, 1976) are not altered with the increasing age of the animals provide firm support to the view that enzymes synthesised in old age are structurally similar to those of young.

From these studies, it can be concluded that the alteration in the levels and regulation of cytoplasmic and mitochondrial NADP<sup>+</sup>-isocitrate dehydrogenase of the liver observed at different phases of life span may be due to the differential template activity of the corresponding gene(s). Thus, the changes at molecular level, brought about by various endogenous factors like hormones may lead to the process of aging in an animal.

### Acknowledgements

The authors are grateful to the Head, Department of Zoology, for providing the necessary facilities. The financial assistance from University Grant Commission, New Delhi is gratefully acknowledged.

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## **Interactions of progesterone with D-amino acid oxidase—Different effects on apo- and holo-enzyme\***

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**Abstract.** A simple method using charcoal treatment was developed for the preparation of apo-D-amino acid oxidase from rat kidney homogenates. This apo-D-amino acid oxidase was used to study the effect of progesterone on the apo- and holo-enzyme. Progesterone inhibited the activity of D-amino acid oxidase, when the apo- enzyme, preincubated with saturating amounts of FAD was used; this effect varied with FAD concentration. Progesterone did not inhibit the activity when added to a mixture of non-preincubated apo-enzyme and FAD; this suggests that progesterone has different effects on apo- and holo D-amino acid oxidase.

**Keywords.** Progesterone; D-amino acid oxidase; rat kidney; flavin adenine dinucleotide.

### **Introduction**

Administration of steroid oral contraceptives containing estrogen and progestogen to adult female rats elevates the activity of some flavin enzymes such as D-amino acid oxidase (D-amino acid: O<sub>2</sub> oxidoreductase (deaminating) EC 1.4.3.3, Ahmed and Bamji, 1976). To elucidate the mechanism of this increase, the *in vitro* interactions of different steroids with highly purified hog kidney D-amino acid oxidase were studied. Tanaka *et al.*, 1978). Among several steroids tested, progesterone inhibited the enzyme markedly, while other compounds were only slightly effective. Similar inhibition of the enzyme activity was observed when progesterone was added to rat liver and kidney homogenates (Ahmed and Bamji, 1977). Subsequent studies with kidney homogenates however showed that the inhibitory effect of progesterone on D-amino acid oxidase activity was abolished and sometimes even stimulation was observed when FAD was added to completely saturate the apo-enzyme present in the homogenate. This observation indicated the possibility of two types of effects of progesterone on the enzyme—an inhibitory effect on the holoenzyme but a stimulatory effect on the apoenzyme. Tissue homogenates contain both holo- and apo-enzymes, and in the presence of added FAD, the stimulatory and inhibitory effects may therefore tend to cancel out each other.

The results of a study to test this possibility presented here suggest that progesterone has different effects on apo- and holo-D-amino acid oxidase.

### **Materials and Methods**

Progesterone, FAD and dextran-T80 were obtained from Sigma Chemical Company, St. Louis, Missouri, USA.

\* Preliminary report presented at the V International Congress of Hormonal Steroids, 29 Oct—4 Nov. 1978, New Delhi. *J. Steroid Biochem.* 9, 832, (abstract 94) 1978.

Charcoal obtained from Merck Chemicals was activated by refluxing with 1N HCl for 8 h. It was then washed several times with water to remove the acid completely and dried. To obtain dextran-coated charcoal, 10 g of charcoal was mixed with 1 g dextran-T80.

#### *Preparation of crude apo-D-amino acid oxidase from kidney homogenates*

Adult female rats of Wistar strain were sacrificed by decapitation, the kidneys removed and homogenised to 20% in glass distilled water.

The following conditions were found to be optimum to obtain apo-D-amino acid oxidase with minimum residual activity and maximum stimulation with added FAD; 2% kidney homogenate (diluted from 20%) was allowed to stand at 0°C for 15 min. Dextran-T80-coated charcoal (5 mg/ml) was added to the homogenate, stirred and immediately centrifuged at 4°C and 1200 g for 30 min. The supernatant was used as a source of the crude apo-D-amino acid oxidase.

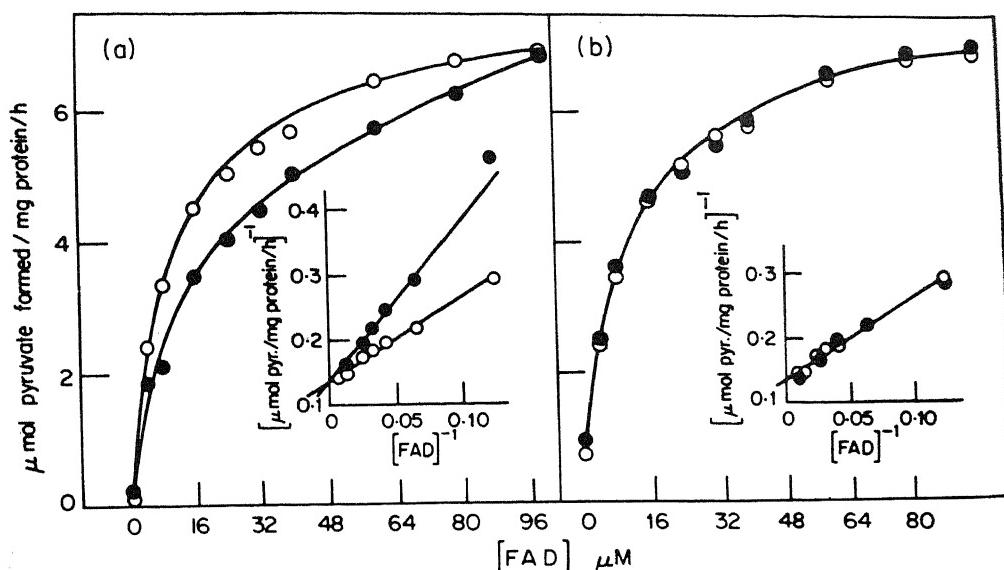
Two types of experiments were carried out. System-1 in which apo-D-amino acid oxidase was preincubated with FAD for 10 min to regenerate the holoenzyme, and progesterone added at the time of starting the reaction with D-alanine. System-2 in which FAD and progesterone were added to the apoenzyme simultaneously, at the time of starting the reaction. A third system involving pre-incubation of apo-enzyme with progesterone and then starting the reactions with FAD and D-alanine was also tried but not pursued since the apo-enzyme was rapidly degraded at 37°C in the absence of FAD. Effects of different concentrations of FAD, D-alanine and progesterone were tested in both the systems.

D-amino acid oxidase activity was assayed by measuring the pyruvate formed from D-alanine (Seifter *et al.*, 1948). Protein was estimated by the method of Lowry *et al.* (1951). The enzyme activity was expressed as  $\mu\text{mol}$  pyruvate formed/mg protein/h.

### Results

The reconstitution of D-amino acid oxidase activity by incubation of apo-enzyme with increasing concentrations of FAD (4 to 100  $\mu\text{M}$ ) in the absence and presence of progesterone (60  $\mu\text{M}$ ) in systems 1 and 2 are presented in figures 1a and 1b. Progesterone inhibited the enzyme activity in competition with the coenzyme, FAD in system 1.  $K_m$  for FAD in the absence and presence of progesterone (60  $\mu\text{M}$ ) being 7.6  $\mu\text{M}$  and 10.1  $\mu\text{M}$  respectively (figure 1a inset). However, at FAD concentrations less than 1  $\mu\text{M}$  progesterone tended to activate the enzyme marginally in system 1 (data not shown).

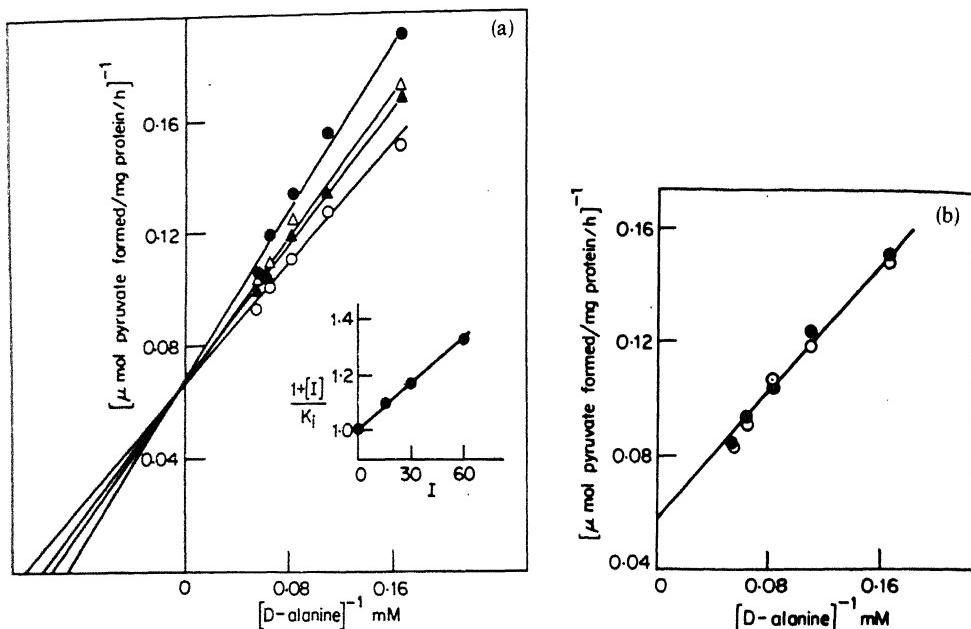
In system 2, where progesterone and FAD (4-100  $\mu\text{M}$ ) were added together to the apo-enzyme, the inhibitory effect seen in system 1 was abolished (figure 1b).



**Figure 1.** Reconstitution of rat kidney D-amino acid oxidase from crude apo-enzyme and FAD and the effect of progesterone. (a) Progesterone effect in system 1. The apoenzyme was preincubated for 10 min with FAD (4-10  $\mu\text{M}$ ) and added before starting the reactions with D-alanine ( $-O-$ ) no progesterone ( $-●-$ ) plus progesterone (60  $\mu\text{M}$ ). (b) Progesterone effect in system 2. Progesterone (60  $\mu\text{M}$ ) and FAD (4-100  $\mu\text{M}$ ) were added together before starting the reaction with D-alanine. The inset shows  $1/v$  vs  $1/(FAD)$  plot with and without progesterone.

The reaction mixture, taken in 15 ml tubes contained: sodium arsenite (0.04 M in 0.11 M NaCl) -0.3 ml; Krebs-Ringer buffer, pH 8.3-1.0 ml; enzyme-0.5 ml; FAD-0.1 ml (4-100  $\mu\text{M}$ ). The reaction was started by the addition of 0.1 ml D-alanine (15  $\mu\text{M}$ ). Oxygen was passed into each tube for 10 sec at a pressure of 50 kg/cm<sup>2</sup> and the tubes closed with rubber stoppers. The reaction was stopped at the end of 1 h with 0.5 ml of 20% trichloroacetic acid. Pyruvate was estimated in the protein-free supernatant, as its 2:4 dinitrophenylhydrozone derivative by a colorimetric method (Seifter *et al.*, 1948). The inset shows double reciprocal plot with and without progesterone.

Figure 2 describes the effects of different fixed concentrations of progesterone on  $1/v$  vs  $1/[S]$  plot of D-amino acid oxidase activity of various concentrations of D-alanine. In system 1 (figure 2a) progesterone produced significant inhibition of the enzyme in competition with its substrate D-alanine. The  $K_m$  for D-alanine was found to be 8.0 mM in the absence of progesterone. The  $K_i$  for progesterone was 1.25  $\mu\text{M}$ .



**Figure 2.** Effect of progesterone on the double reciprocal plot of catalytic oxidation of D-alanine by rat kidney D-amino acid oxidase reconstituted from the apo-enzyme and FAD (60  $\mu\text{M}$ ), in presence of varying concentrations of D-alanine.

The concentrations of progesterone used were, none (O), 15  $\mu\text{M}$  ( $\blacktriangle$ ) 30  $\mu\text{M}$  ( $\Delta$ ) and 60  $\mu\text{M}$  ( $\bullet$ ). Progesterone was added in 10  $\mu\text{l}$  of alcohol to obtain the desired concentration. Same amount of alcohol was added to control tubes. Linear best fit pots were obtained by the method of least squares.

(a). Progesterone effect in system 1 (as in legend to figure 1)

(b). Progesterone effect in system 2 (as in legend to figure 1)

—○— no progesterone; —●— + progesterone (60  $\mu\text{M}$ )

Inset in figure 'a' shows plot of  $1 + [I]/K_i$  vs  $[I]$  where  $K_i$  is the dissociation constant for the enzyme-inhibitor complex, and  $[I]$  is the concentration of progesterone.

## Discussion

The effects of progesterone on the regenerated holo-D-amino acid oxidase (system 1) at various D-alanine concentrations (figure 2a) are analogous to the observations of Tanaka *et al.* (1978) with the highly purified hog kidney enzyme. The  $K_i$  (1.25  $\mu\text{M}$ ) observed in the present study is lower than (18  $\mu\text{M}$ ) observed by Tanaka *et al.* (1978). This difference may probably be due to the difference in the sources of the enzyme or due to the environment provided in the crude system.

Tanaka *et al.* (1978) postulated that inhibition of D-amino acid oxidase by progesterone may be due to a steric block of the substrate binding site by progesterone. Since progesterone increased fluorescence emission as well as fluorescence polarisation of the enzyme, the authors ruled out the possibility that progesterone was causing dissociation of FAD from the enzyme. However, the data presented in figure 1a

show that progesterone increased  $K_m$  for FAD in system 1, suggesting that the hormone does affect FAD binding at FAD concentrations greater than 4  $\mu\text{M}$ . In view of the fact that in the enzyme, the binding sites for substrate and coenzyme are in close proximity it is possible that progesterone produces a steric block of FAD binding site in the holo-enzyme and thus prevents reassociation of FAD which is removed during enzyme catalysis.

The opposite effects of progesterone at high concentrations of FAD in system 1 and at all concentration of FAD in system 2 (where FAD, progesterone and substrate were added simultaneously to apoenzyme) are difficult to explain. It is possible that progesterone binds to different sites on apoenzyme and holoenzyme, producing different effects. In the holoenzyme, progesterone may produce a steric block of substrate and FAD binding sites and inhibit the activity. Binding of progesterone to the apoenzyme may produce changes which facilitate association with FAD and eliminate the steric block. Preliminary studies of Tanaka and Yagi (personal communication) with purified hog kidney D-amino acid oxidase also suggest that progesterone may increase the association of FAD with the apo-enzyme. The possibility of these two different types of effects of progesterone on apo- and holo-enzymes derives support from the observation that when FAD concentration was very low (permitting most of the enzyme to be in apoenzyme form) progesterone produced slight activation of the enzyme even in system 1.

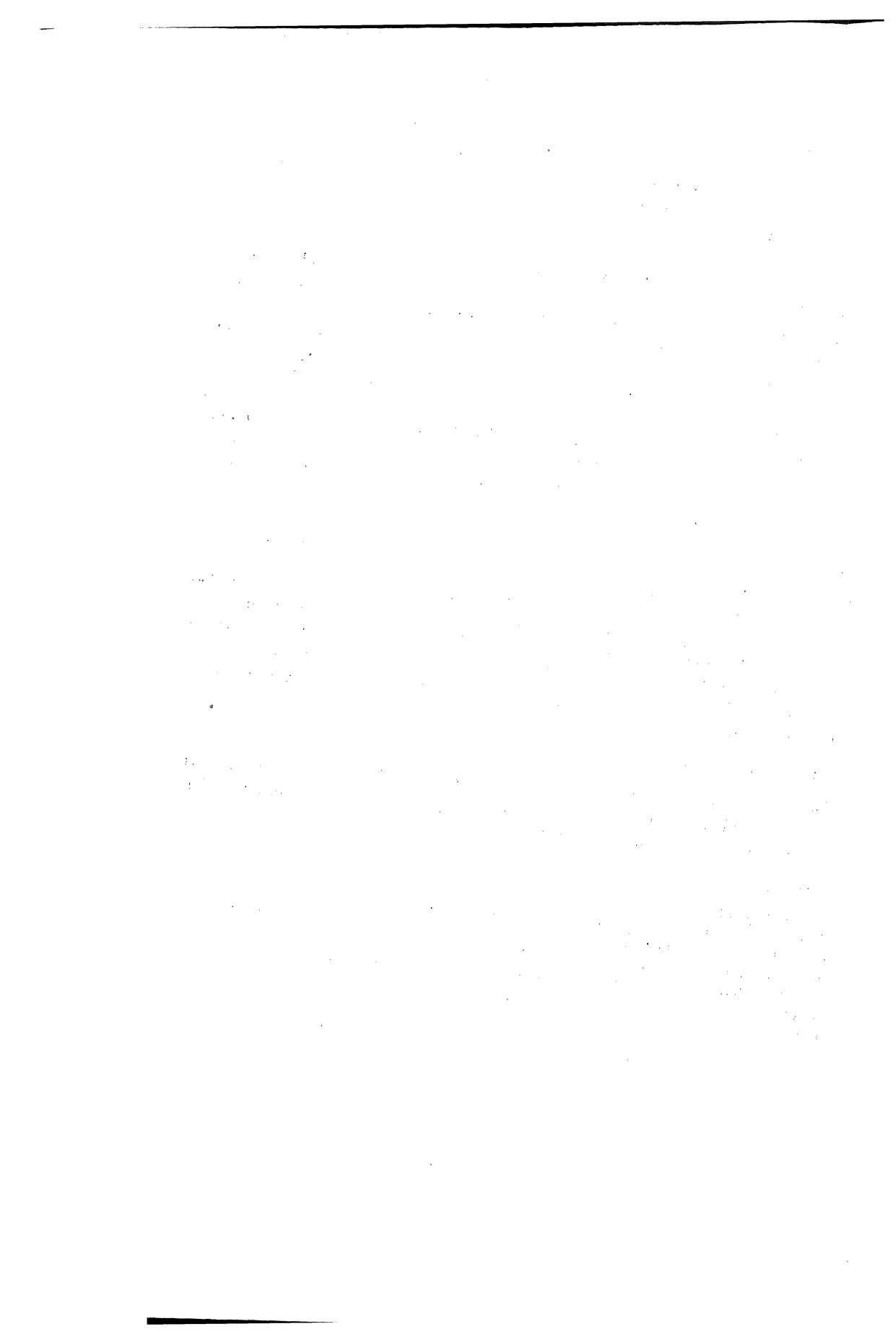
The physiological significance of the dual effects of progesterone on D-amino acid oxidase is not clear, since it is an enzyme of limited biological significance and the observed changes are of a small magnitude. The latter however is not surprising in view of the opposite effects. Such hormonal effects if observed for other enzymes may have significance in the regulation of enzyme activities at low and high concentrations of coenzyme.

### Acknowledgements

The authors are grateful to Dr. S. G. Srikantia, Director, National Institute of Nutrition, Hyderabad and to Dr. N. Appaji Rao, Department of Biochemistry, Indian Institute of Science, Bangalore for the benefit of useful discussions.

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## Lectin from rice

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MS received 21 August 1979; revised 9 January 1980

**Abstract.** N-Acetyl-D-glucosamine-binding lectin was isolated and purified from rice by ammonium sulphate fractionation and affinity chromatography using N-acetyl-D-glucosamine linked Sepharose 6B column. It gave a single band on polyacrylamide disc gel. It was identified as a glycoprotein. The purified lectin dissociated into two components on Sephadex G-100 column chromatography,—a higher molecular weight fraction not containing any carbohydrate and a lower molecular weight glycoprotein fraction. The apparent molecular weights of these fractions were 85,000 and 14,500. The lectin agglutinated erythrocytes of human A,B,O groups and of several other mammals and its activity was inhibited only by N-acetyl-D-glucosamine. The glycopeptide isolated by pronase digestion of the lectin was homogeneous and did not possess agglutinating activity. It contained about 10% carbohydrate of which xylose, arabinose and glucose were the major components.

**Keywords.** Rice lectin; affinity chromatography; glycoprotein; glycopeptide; reversible hemagglutination.

## Introduction

Lectins are carbohydrate binding proteins. These agglutinate erythrocytes and other types of cells (Sharon and Lis, 1972). Lectins have been used in the studies of membrane structure, function and cell transformation (Lis and Sharon, 1973; Nicolson, 1974; Rapin and Burger, 1974). Purification and properties of lectins have been reviewed in detail by Goldstein and Hayes (1978). Since many lectins have affinity towards sugars, this property of the lectins has been utilised for their purification by affinity chromatography. A special account of N-acetyl-D-glucosamine-binding lectins has been dealt by Caderburg and Gray (1979). The present study deals with the isolation, purification by affinity chromatography and describes a few properties of an N-acetyl-D-glucosamine binding lectin from an Indian variety of rice.

## Materials and methods

### Materials

Rice, S-701 and other varieties, used in these experiments were obtained from Visweswaraiah Canal Farm, Mandya, Karnataka.

Epoxy-activated Sepharose 6B and Sephadex G-100 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. N-Acetyl-D-glucosamine, other sugars used, bovine serum albumin, ovalbumin, trypsin and lysozyme were purchased from Sigma Chemicals Co., St. Louis, Missouri, USA. Pronase was a Kaken Chemicals' product, Tokyo, Japan. All the other chemicals used were of the analytical grade.

Human A,B,O group erythrocytes were obtained from the Blood Bank of the local hospital. Erythrocytes of other experimental animals were collected from the animal house, Central Food Technological Research Institute, Mysore.

### Methods

*Synthesis of N-acetyl-D-glucosamine Sepharose 6B:* This biospecific adsorbant was prepared according to the procedure of Vretblad (1976) by coupling N-acetyl-D-glucosamine to epoxy-activated Sepharose 6B.

*Preparation of the crude extracts of rice:* Ten different varieties of rice were screened for the hemagglutinating activity by the following procedure. Cleaned paddy was dehusked and the rice obtained was powdered and passed through an 85-mesh sieve. The flour was defatted by repeated extraction with cold petroleum ether and air-dried. Defatted rice flour was dispersed in 0.05 M sodium citrate buffer of pH 4.0 in the ratio of 1:5 (w/v), stirred in cold for 3 h and centrifuged at 2000 g for 20 min. The clear supernatant obtained was dialysed against phosphate-buffered-saline (PBS), pH 7.4 (0.006 M) and checked for the hemagglutinating activity.

*Isolation of the crude rice lectin from S-701 variety:* Crude lectin was precipitated by adding solid ammonium sulphate to 50% saturation to the supernatant prepared as described above from S-701 variety rice. The precipitate recovered by centrifugation was dissolved in a minimum amount of 0.05 M sodium acetate buffer, pH 4.5 and dialysed extensively against the same buffer.

*Purification of the lectin on the biospecific adsorbant:* Fifteen of N-acetyl-D-glucosamine-linked Sepharose 6B was packed in a column (16 × 60 mm) and was equilibrated with 0.05 M sodium acetate buffer, pH 4.5. Four ml of the crude lectin (2.5 mg/ml) was loaded and allowed to flow through the column at a rate of 10 ml/h which was maintained throughout the experiment. The unadsorbed protein was washed with 0.05 M acetate buffer (pH 4.5) till the effluent showed no absorbance at 280 nm. The bound lectin was then eluted from the column with N-acetyl-D-glucosamine (60 mg/ml). The emergence of the lectin was monitored by measuring absorbance at 280 nm. Appropriate fractions were pooled, concentrated by ammonium sulphate precipitation at 50% saturation, dissolved in a minimum amount of phosphate-buffered-saline and dialysed extensively against the same buffer.

*Hemagglutination assay:* Hemagglutination assay was carried out by the serial dilution technique with trypsinized rabbit-erythrocytes (Lis and Sharon, 1972). The highest dilution of the lectin causing visible agglutination was identified as the titre value. The hemagglutinating activity of the lectin against trypsinized erythrocytes of different species was examined by adopting the same procedure.

*Polyacrylamide disc gel electrophoresis:* Electrophoresis was carried out in 7.5% gels using 0.005 M Tris-glycine buffer, pH 8.3, according to the method of Ornstein (1964). The gels were stained with 0.1% amido black in 7% acetic acid and destained in 7% acetic acid solution. The presence of the glycoprotein was detected by staining the gels with periodic acid-Schiff's reagent and destaining by using 1% sodium metabisulphite.

*Molecular weight determination:* The molecular weight of the purified rice lectin was determined from the data obtained by gel filtration on Sephadex G-100. Bovine serum albumin, ovalbumin, trypsin and lysozyme were used as the reference proteins. Sephadex G-100 column ( $1.5 \times 81$  cm) was equilibrated with phosphate-buffered saline. Four ml of the purified lectin (2 mg/ml) in the buffer was loaded on to the column and eluted with the same buffer. Fractions (2 ml) were collected at a flow rate of 15 ml/h. Protein absorbance was measured at 280 nm. Elution of the other proteins was similarly carried out. A calibration curve was obtained by plotting log molecular weights of the reference proteins vs their  $V_e/V_0$  values.

*Sugar inhibition studies:* Inhibition of hemagglutination by various sugars was tested as follows. The purified lectin (5 µg) in 0.1 ml of phosphate-buffered-saline was serially diluted with the same buffer. To each of the dilutions 0.1 ml of the test-sugar was added and incubated for 1 h at 37°C. Later 0.2 ml of 2% trypsinised-rabbit-erythrocytes were added, kept at 37°C for 1 h and observed for hemagglutination. Reversibility of hemagglutination by the inhibitory sugar (N-acetyl-D-glucosamine) was performed according to the procedure of Lis *et al.* (1970).

*Preparation of the glycopeptide:* The lectin was digested exhaustively with pronase in the procedure of Kawai and Anno (1975). The undigested protein and the added enzyme were precipitated with trichloroacetic acid and removed by centrifugation. The glycopeptide was precipitated from the supernatant by adding four volumes of 10% alcohol in the presence of 1% potassium acetate (Kirk and Derby, 1957). It was repeatedly washed with alcohol and dried *in vacuo*.

*Sugar composition of the glycopeptide:* The total carbohydrate content of the glycopeptide was estimated by phenol-sulphuric acid reagent (Dubois *et al.*, 1956).

#### *Qualitative examination of sugars*

The sugar composition of the glycopeptide was determined by hydrolysing 2 mg of it with 2 ml of 2 N sulphuric acid for 6 h in a boiling water-bath. It was neutralised with barium carbonate and fractionated on Dowex-50 ( $H^+$ ) and Dowex-2 (formate) columns (Simkin *et al.*, 1964). The neutral sugar fraction was eluted with water and concentrated *in vacuo* at 40°C to remove HCl. The neutral and aminosugars were identified by paper using *n*-butanol:pyridine:water system (6:4:3 v/v). The chromatograms were developed for 36 h and sprayed separately with alkaline silver nitrate to identify the neutral sugars and alkaline acetyl acetone reagent for aminosugars.

#### *Quantitative estimation of neutral sugars*

Quantitative estimation of neutral sugars was carried out after their separation by chromatography on freshly prepared potato starch column (Gardell, 1953). The column ( $0.9 \times 26$  cm) was equilibrated with 200 ml of *n*-butanol:*n*-propanol:water 4:1:1 (v/v). The neutral sugar fraction obtained from 9 mg of glycopeptide was loaded on to the column and eluted with the above solvent mixture. Fractions (1 ml) were collected. One-half of each fraction was evaporated separately *in vacuo* and analysed for sugar by Nelson-Somogyi method (Nelson, 1944; Somogyi, 1952). The fractions containing individual sugars were pooled, evaporated to dryness and identified by paper chromatography to be xylose, arabinose and glucose. The elution pattern of the test sample was compared with that of the standard sugar mixture containing the sugars suspected to be present in the test sample.

## Results and discussion

### *Hemagglutinating activity in the crude extracts of Indian varieties of rice*

The presence of lectin in a Japanese variety of rice (*Akibare*) was first reported by Takahashi *et al.* (1975). These workers purified the lectin by ammonium sulphate fractionation and ion-exchange chromatography on DEAE- and CM-celluloses. Its molecular weight, amino acid composition, sugar composition etc., were studied. However, they have not reported on the sugar inhibition of hemagglutination by the lectin.

Extracts of several Indian varieties of rice were examined for the presence of hemagglutinating activity, of which S-701 variety contained the maximum activity (table 1). The lectin from this variety of rice was partially purified by adding ammonium sulphate to the extract to 50% saturation. The activity of this crude lectin was specifically inhibited only by N-acetyl-D-glucosamine. This affinity of the lectin for this sugar was utilised for its further purification.

**Table 1.** Hemagglutinating activity of the crude extracts of different Indian varieties of rice.

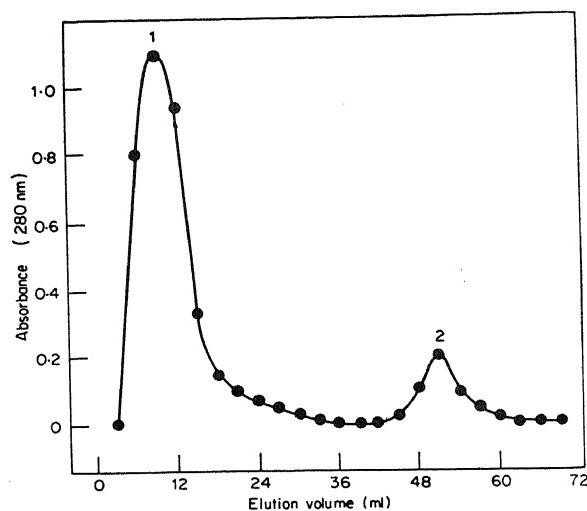
Variety tested	Titre value/ 0.2 ml of the crude extract
IET-2724	1
IET-2730	1
S-701	4

Hemagglutinating activity was not present in C-435, CH-45, IET-3232, Mahsuri, MR-301, Pushpa, SR-26B and Sona varieties of rice.

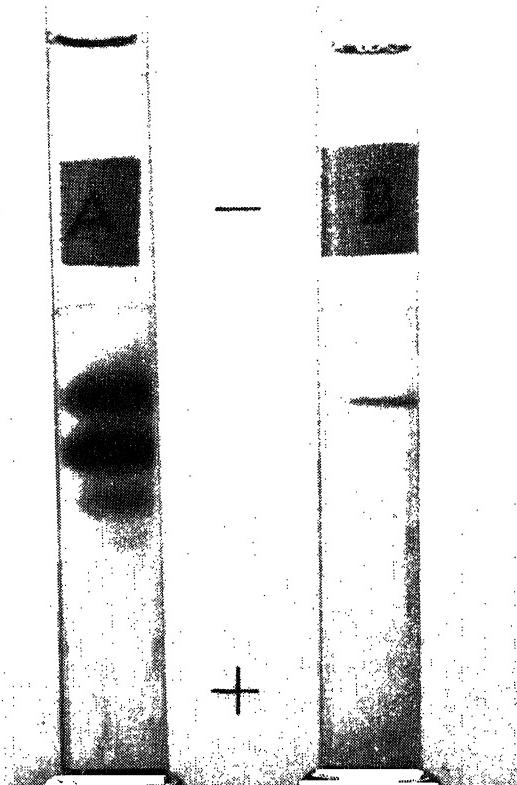
### *Purification of lectin*

When the crude lectin was passed through the N-acetyl-D-glucosamine-Sepharose 6B column, the lectin was bound to the biospecific adsorbant. This was shown by the absence of activity in the unadsorbed portion of the protein removed by washing with 0.05 M acetate buffer, pH 4.5. The bound-lectin could be eluted by sodium acetate buffer containing N-acetyl-D-glucosamine yielding a pure protein (peak 2, figure 1).

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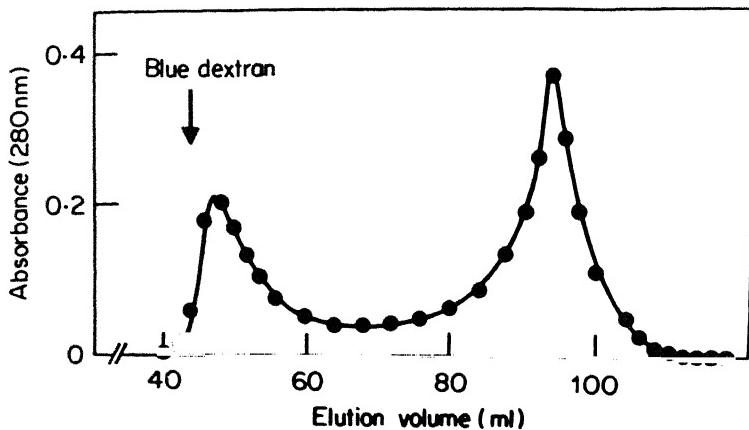
**Figure 1.** Affinity chromatography of the crude rice lectin. 4 ml of the crude lectin (2.5 mg/ml) was applied to N-acetyl-D-glucosamine linked Sepharose 6B column and was washed with 0.5 M acetate buffer of pH 4.5 at a flow rate of 10 ml/h till the eluant showed no absorbance at 280 nm. The column was eluted with N-acetyl-D-glucosamine (60 mg/ml) in the same buffer. Peak 1 denotes the unadsorbed protein and peak 2 the purified rice lectin.



**Figure 2.** Polyacrylamide gel electrophoretic pattern of rice lectin. A. Crude rice lectin (50% ammonium sulphate fraction). B. Lectin purified by affinity chromatography on N-acetyl-D-glucosamine-linked Sepharose 6B column.

### Dissociation and molecular weight of the lectin

The purified lectin eluted as two peaks from Sephadex G-100 column using phosphate-buffered-saline as the eluant and the activity was found in both the fractions (figure 3). This anomalous behaviour was also observed in the case of ulex and lotus lectins



**Figure 3.** Sephadex G-100 column chromatography of the purified rice lectin. 8 mg of the pure lectin in 4 ml of phosphate-buffered-saline was applied to a Sephadex G-100 column ( $1.5 \times 81$  cm). The column was eluted with phosphate-buffered-saline at a flow rate of 15 ml/h. 2 ml fractions were collected and absorbance measured at 280 nm. Peak 1 noncarbohydrate and peak 2 carbohydrate containing fractions.

(Allen and Johnson, 1977). The apparent molecular weights of the two fractions (as determined on G-100 column calibrated with molecular weight markers-lysozyme, trypsin, ovalbumin and bovine serum albumin) were 85,000 and 14,500 respectively. The lower molecular weight protein contained the carbohydrate moiety as shown by the phenol-sulphuric acid test. Takahashi *et al.* (1973) reported a molecular weight of 10,000 for the rice lectin from *Akibare* variety which was a glycoprotein. In this respect, the lectin from S-70 is interesting in that, a part from the lower molecular weight glycoprotein fraction, it contains a higher molecular weight non-glycoprotein fraction.

### Effect of carbohydrate on the hemagglutinating action

The lectin agglutinated trypsinized and untrypsinized erythrocytes of rabbit, rat, brown mice, hamster, guinea pig, monkey and human A,B,O blood groups.

The agglutinating activity of the lectin against trypsinized erythrocytes of rabbit was not inhibited by D-xylose, D-arabinose, D-fucose, L-fucose, D-glucose, D-galactose, D-mannose, D-glucosamine, D-galactosamine, N-acetyl-D-galactosamine, N-acetyl neuraminic acid, maltose, lactose, sucrose, melibiose and melizitose even at 100 mM concentration. Only N-acetyl-D-glucosamine could inhibit the agglutination at 1 mM concentration (table 2).

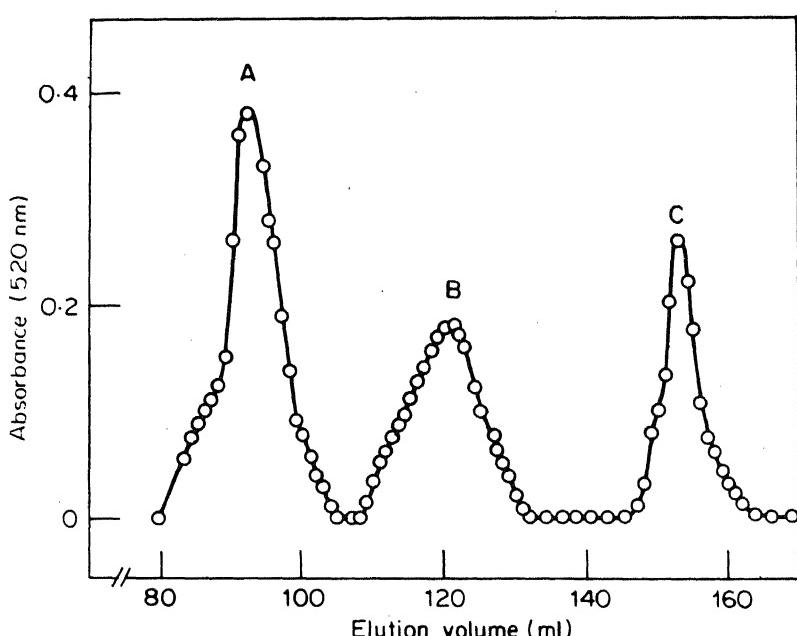
The agglutinated-rabbit-erythrocyte clot could be reversed completely only by N-acetyl-D-glucosamine at 300 mM concentration (data not shown).

**Table 2.** Inhibition of haemagglutination by sugars.

Test sugar	Sugar concentration (mM)	Titre
Lectin alone	—	64
D-glucosamine	100	64
D-galactosamine	100	64
N-acetyl neuraminic acid	100	64
N-acetyl-D-galactosamine	100	64
N-acetyl-D-glucosamine	1	16
	10	8
	100	0

*Preparation and sugar composition of the glycopeptide*

The glycopeptide prepared by pronase-digestion of the lectin was homogeneous on polyacrylamide gel electrophoresis. It had no agglutinating activity against rabbit erythrocytes. On the other hand, the lectin digested by chymotrypsin for 24 h did not show significant loss of activity. These experiments suggest that extensive digestion of the lectin resulted in the loss of activity whereas limited breakage of the peptide bonds does not affect the activity.



**Figure 4.** Column chromatography of neutral sugars of the glycopeptide. Neutral sugar fraction obtained from 9 mg of the glycopeptide was applied to a starch column and eluted with *n*-butanol:*n*-propanol:water mixture (4:1:1 v/v) at a flow rate of 2 ml/h. 1 ml fractions were collected. Peaks A, B and C represent xylose, arabinose and glucose respectively.

The glycopeptide contained about 10% carbohydrate. Xylose, arabinose and glucose constitute the neutral sugar fraction as shown by paper chromatography. No aminosugars were detected by paper chromatography. The three neutral sugars could be separated and estimated quantitatively on a starch column (figure 4).

**Table 3.** Carbohydrate composition of the glycopeptide prepared from rice lectin.

Sugar	mol/100 mg of the glycopeptide
Xylose	27.8
Arabinose	22.2
Glucose	11.1

Xylose, arabinose and glucose were present in the molar ratio of 2.5:2.1 (table 3). Takanashi *et al.* (1973) reported that the rice lectin they prepared contained glucose, mannose, xylose and glucosamine in the ration of 13:1:1:1. In this respect also, the lectin from variety S-701 was different in that it lacked mannose and glucosamine but contained arabinose. Thus the lectin from S-701 seemed to be primarily a pentose containing glycoprotein.

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## **Impact of malathion on acetylcholinesterase in the tissues of the fish *Tilapia mossambica* (Peters)—A time course study**

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MS received 26 November 1979; revised 19 February 1980

**Abstract.** The sublethal toxic potency of malathion in inhibiting acetylcholinesterase activity of brain, muscle, gill and liver tissues of the fish, *Tilapia mossambica* was studied at 12 h intervals. Maximum inhibition at 36 and 48 h, and complete revival of acetylcholinesterase activity after 72 h was noticed, suggestive of the loss of inhibition of the enzyme activity was probably by suitable (acetylcholine) accumulation.

**Keywords.** Malathion; acetylcholinesterase; acetylcholine; *Tilapia mossambica*.

### **Introduction**

Malathion, a widely used insecticide is known to cause serious metabolic disturbances in non-target species, like fish and fresh-water mussels (U.S.E. Prot. Agen. 1972). Malathion is known to affect the nervous system by inhibiting acetylcholinesterase (AChE), the enzyme that modulates the amount of the neurotransmitter acetylcholine (Fukuto, 1971). There are several metabolic routes by which an organism can detoxify organophosphorus insecticides. In addition, The physiological condition of the organism during toxic impact must be considered to understand the influence of pesticide. In the present study malathion was chosen to evaluate its influence on the acetylcholinesterase activity and acetylcholine content in the tissues of the fish *Tilapia mossambica* at different times after exposure to malathion.

### **Materials and methods**

The fish, *Tilapia mossambica* was collected from streams around Tirupati and acclimated to laboratory conditions for about a week. They were fed daily with groundnut-cake (solid material obtained after extracting oil from the seeds) and frog muscle twice a week. Technical grade malathion of 95% purity was used. The lethal concentration for 50% killing ( $LC_{50}$ ) values was computed on the basis of probit analysis (Finney, 1964) and was found to be 5.6 ppm. Two ppm malathion was chosen to represent sublethal concentration. Malathion was added only once and the fish tanks were aerated at 12 h intervals.

Fishes weighing  $8 \pm 2$  g were separated into 7 batches of 10 each. One batch of fishes were exposed to 80 litres of tap water in two 50 litres glass-tanks. The remaining 6 batches of fishes were exposed to 480 litres of 2 ppm malathion in tap-water in 12 glass tanks. The period of exposure was adjusted to obtain fishes at two time-inter-

vals exposure per day. Acetylcholinesterase and acetylcholine levels were estimated every 12 h. Since there was no significant change in the value of controls at different periods, only average values are represnted in the results choosing the value for one fish at each interval.

After exposure of the fish to malathion, brain, muscle, gill and liver were removed for homogenisation in cold 0.25M sucrose. Acetylcholine and the esterase activity were determined by the method of Metcalf (1951). Protein was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

### Results and discussion

The changes in acetylcholinesterase activity in the brain, muscle, gill and liver tissues of malathion-exposed fishes decreased significantly upto 60 h. Maximal inhibition of the enzyme was observed at 36 and 48 h intervals (table 1). Concomitant with the decreased AChE activity, the ACh content correspondingly increased after exposure for 36 and 48 h (table 2).

Since the inhibition of acetylcholinesterase activity gradually increased from 12 to 36 and 48 h and decreased fro 48 to 72 h (table 1), it can be suggested that inhibition of the esterase by malathion is dependent on the duration of exposure. After 72 h of exposure, the enzyme activity in all the tissues as comparable to that of normal tissues, thus indicating the reversal of inhibition in treated fishes. It is likely that the effect of malathion decreases after 48 h probably due to its degradation.

The differential inhibition of acetylcholinesterase activity in the four tissues (brain > muscle > gill > liver) may be due to the presence of isozymes with different affinities for the substrate and the inhibitor. Further, the pesticide itself may be present in different amounts in the different tissues producing differential inhibition or the inhibitor may be metabolised at different rates.

Corresponding to maximal inhibition of acetylcholinesterase activity at 36 and 48 h of exposure, acetylcholine content also showed a corresponding increase (data not a substrate of the esterase, the changes observed in its content in the four tissues at acetylcholine. During 72 h when the esterase activity was restored to the normal level the acetylcholine content also returned to normal levels. Since actylcholine is a substrate for the esterase, the changes observed in its content in the four tissues at different times of exposure were compatible with alterations in the enzyme activity.

It is well known that the inhibitory effects of malathion result from the relatively longer half life of the phosphorylated enzyme, as compared with the acetylated enzyme of the physiological reaction (Wilson, 1967; Albridge, 1971). Since there was only a single addition of malathion to the water without any change of water, it is likely that the phosphorylation of the enzyme may have occurred only upto 48 h and later the amount of the phosphorylated enzyme could have decreased due to lowered levels of the pesticide.

It is evident from our earlier findings, that malathion inhibits acetylcholinesterase in four tissues of *T. mossambica*, competitively (Kabeer and Ramana Rao, 1980). In the presence of high amounts of acetylcholine, the inhibitory effect produced by malathion is known to be reduced (Augustinsonn and Nachmansohn, 1949). Thus the accumulation of high concentrations of acetylcholine during 36 and 48 h may contribute towards reversal of the inhibition of enzyme activity. However, the possibility is rather doubtful since acetylcholine can compete for the free enzyme and not for the already phosphorylated enzyme. The most possible explanation is that the accumulated acetylcholine may induce the formation of increased amounts of acetylcholinesterase leading to the revival of affected fishes.

**Table 1.** Acetylcholinesterase (AChE) activity of brain, muscle, gill and liver tissues of normal and malathion exposed fishes at different hours of exposure.

Tissue	Control	Specific activity of AChE : $\mu$ mol/mg protein h <sup>-1</sup>					Hours of malathion exposure	
		12	24	36	48	60		
Brain	4.8±0.2	M	3.9	2.6	2.3	2.4	4.1	4.7
	S.D.	±0.2	±0.4	±0.3	±0.2	±0.3	±0.3	±0.3
	PD	-19.1 <i>P</i> <0.001	-46.2 <i>P</i> <0.001	-50.7 <i>P</i> <0.001	-49.3 <i>P</i> <0.001	-15.3 <i>P</i> <0.001	-1.9 N.S.	-1.9 N.S.
Muscle	3.6±0.2	M	3.0	2.5	1.9	1.8	3.0	3.8
	S.D.	±0.3	±0.3	±0.1	±0.2	±0.4	±0.3	±0.3
	PD	-17.2 <i>P</i> <0.001	-31.5 <i>P</i> <0.001	-47.4 <i>P</i> <0.001	-48.3 <i>P</i> <0.001	-15.5 <i>P</i> <0.025	-5.4 N.S.	-5.4 N.S.
Gill	2.4±0.1	M	2.0	1.8	1.4	1.4	2.0	2.4
	S.D.	±0.1	±0.2	±0.2	±0.2	±0.2	±0.1	±0.1
	PD	-15.4 <i>P</i> <0.001	-28.1 <i>P</i> <0.001	-40.6 <i>P</i> <0.001	-41.9 <i>P</i> <0.001	-17.4 <i>P</i> <0.05	-1.0 N.S.	-1.0 N.S.
Liver	1.4±0.1	M	1.2	1.1	0.9	0.0	1.2	1.4
	S.D.	±0.1	±0.1	±0.8	±0.2	±0.1	±0.0	±0.0
	PD	-14.0 <i>P</i> <0.05	-22.1 <i>P</i> <0.001	-34.3 <i>P</i> <0.001	-37.5 <i>P</i> <0.001	-16.6 <i>P</i> <0.025	-2.7 N.S.	-2.7 N.S.

Each value is the mean ± S.D. of 6 individual observations. PD=Percent deviation from normal. *P*=‘t’ test. N.S.=Not significant.

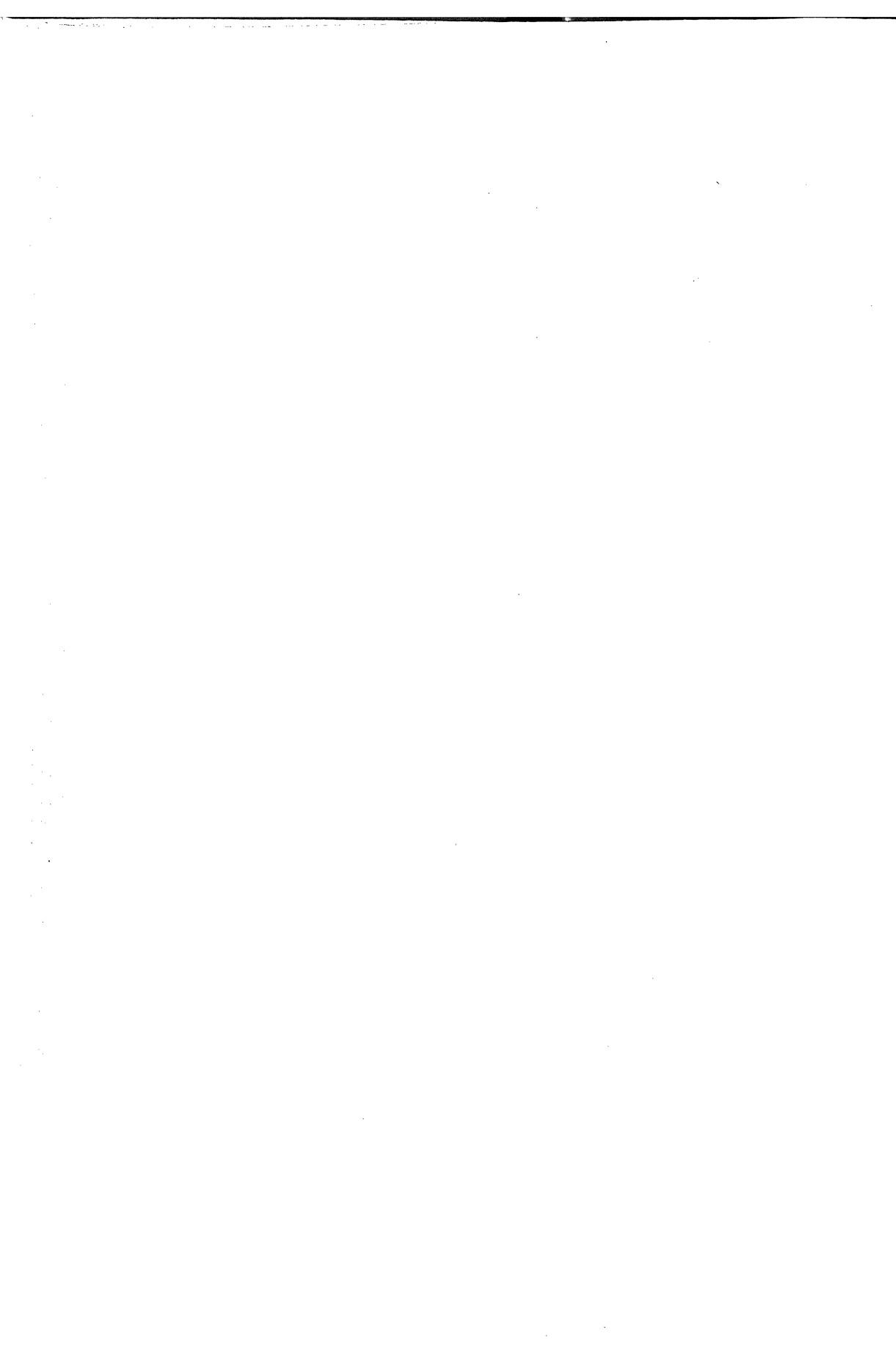
**Table 2.** Acetylcholine content of brain, muscle, gill and liver tissues of control and melathion exposed (ME) fishes at different hours of exposure.

Tissue	Control	ACh content ( $\mu$ mol/g wet wt)					
		Hours of melathion exposure					
		12	24	36	48	60	72
Brain	43.4±4.9	M	56.3	62.7	73.7	73.8	58.0
	S.D.	±3.7	±2.4	±3.7	±4.6	±4.5	45.1 ±4.1
	PD	+29.6	+44.2	69.6	+69.7	+33.4	+3.7
Muscle	33.9±3.0	M	44.5	53.5	60.0	57.6	41.8
	S.D.	±3.9	±3.8	±2.7	±2.8	±2.7	37.8 ±2.8
	PD	+3.1	+57.6	+76.9	+69.6	+23.1	+11.5
+31.1							
							$P < 0.025$
Gill	27.6±3.1	M	33.2	45.9	55.2	54.2	39.1
	S.D.	±2.3	±2.7	±3.2	±3.3	±2.5	31.5 ±2.3
	PD	+20.3	+66.3	+99.9	+97.7	+41.6	+14.1
Liver	24.4±2.1	M	33.2	46.1	50.0	41.5	32.1
	S.D.	±2.6	±2.7	±2.2	±3.2	±2.5	26.6 ±1.6
	PD	+35.7	+88.3	+104.3	+69.7	+31.2	+4.4
							$P < 0.025$

Each value is the mean ±S.D. of 6 individual observations. P.D. = Percent deviation from normal,  $P = t$ 's test.  
N.S. = Not significant.

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## Studies on the oxidation of tannins by *Aspergillus flavus*

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MS received 14 December 1979; revised 14 February 1980

**Abstract.** A method for the estimation of tannin in presence of catechin, pyrogallol, protocatechuic acid and gallic acid using polyamide column chromatography was developed. Tannin added to the growing culture of *Aspergillus flavus* was oxidised to different extents depending on the duration of incubation. The oxidised compound was identified in the culture filtrate as a polymerised product of tannin.

**Keywords.** Tannins; nontannins; tannin precipitation; polyamide adsorption; polymerisation; gel filtration.

### Introduction

A large number of methods for the determination of tannins and related polyphenols have appeared in the literature. However, none is accurate enough to determine the tannin in presence of non-tannins at a very low concentration. The volumetric permanganate method of Lowenthal (1877) and the colorimetric method of Rosenblatt Peluso (1941) and Pro (1952) have the limitation that the total phenolic hydroxyl groups present in both tannin and non-tannin are estimated. Routinely, hide powder is used to remove the major part of the simple phenols and the bulk of the other major complex materials. In the present study, polyamide has been tried for the adsorption of tannins in presence of nontannins and found to be much more efficient than hide powder.

Natural plant tannins represent a group of high molecular weight polyphenolic materials which are widely distributed throughout the plant kingdom (Hathway, 1962; Turd, 1962). They are generally considered to be resistant to microbial decomposition or polymerisation. Condensed tannins, however are more resistant than hydrolysable tannins (Lewis, 1966; Benoit, 1966). Filamentous fungi, especially, species of the genera *Penicillium* and *Aspergillus* have been implicated in the decomposition of tannin (Nishira and Mugibayashi, 1953, 1956). The production of keto acids (Beveridge and Hugo, 1964; Dykerhoff and Armbruster, 1933; Freudenberg and Vollbrecht, 1921) and of oxalic acid (Hathway, 1962) by degradation of phenolic compounds during the decomposition of tannins were observed. In contrast, Hathway (1958) and Hathway and Seakins (1957) identified the oxidative polymers of the accompanying catechins in which C—C links through the 2 phenyl group appeared to be the main mode of condensation.

The condensed tannins which are used as tanning materials in the leather industry, are normally polymerised by molds and are also decomposed by soil microorganisms. It was therefore of interest to investigate the action of *Aspergillus flavus*,

isolated from soils contaminated with tannery wastes, on condensed tannins and develop a method for estimating tannins.

### Materials and methods

#### Materials

Phloroglucinol, gallic acid, polyamide were purchased from E. Merck, Darmstadt, Germany. Protocatechuic acid and cinchonine sulphate were obtained from Koch-Light Laboratories Ltd., Colnbrook, England and from B.D.H. Chemicals Ltd., Poole, England, respectively. Catechin and rutin were purchased from Zyma, S.A., Nyon, Switzerland. Tannic acid was obtained from Fluka Ag Buchs SO, Switzerland. Caffeine was isolated from coffee seeds following the method of Sondheimer *et al.* (1961). All other chemicals were of reagent grade.

#### Methods

*Preparation of pure tannin from wattle extract:* To a 5% solution of spray-dried wattle extract, a saturated solution of caffeine was added until the precipitation was complete. The solution was centrifuged, the precipitate was collected and dried under vacuum. The dry material was dissolved in methanol and precipitated with 5 volumes of chloroform. The precipitate was collected under nitrogen. The process was repeated twice. The purity of the tannin powder was tested by thin layer chromatography using butanol; acetic acid; water (4:1:5) solvent system and by spraying with diazotised p-nitroaniline reagent.

*Estimation of phenolic compounds and tannin:* Standard curves for tannin and other phenolic compounds were constructed using the method of Folin and Ciocalteu (1927). One ml solution of tannin, gallic acid, catechin, protocatechuic acid and phloroglucinol (concentration indicated in figure) in the medium (described under growth condition) 7.0 ml of distilled water was added and the contents were mixed with 1.5 ml of 20% sodium carbonate and 0.5 ml of folin-phenol reagent. The mixture was shaken well, kept at room temperature for 1 h and absorbance was measured at 725 nm in a SP Unicam 1800 spectrophotometer.

*Estimation of tannin-phenolics by precipitation:* Tannins (0.1-2.5 mg/ml) were precipitated by adding either cinchonine sulphate, caffeine and or lead acetate solutions (Peri and Pompi, 1971); Suntharkar and Jatkar, 1938). The concentrations of the reagents required for completely precipitating the tannins were determined earlier. The precipitate containing tanin phenolics was dissolved in a known volume of 10% methanol and total phenolic OH groups estimated (Folin and Cicocalteu, 1927).

*Estimation of tannin by polyamide adsorption method:* Tannin solution (2.5 mg) was adsorbed on to a column of polyamide (1.5×4 cm) equilibrated with 90% methanol. The adsorbed material was eluted successively with 90, 60, 30 and 10% methanol (at a flow rate of 50 ml/h). The total phenolic OH groups were estimated in the eluate as described above.

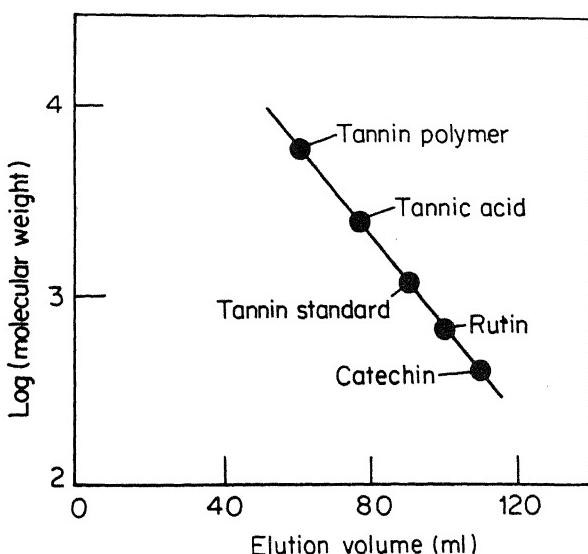
*Organism and growth conditions:* The mold *A. flavus* was isolated from the soil collected from tannery wastes disposal area (Mallika *et al.* 1980). The growth medium containing 1 g KH<sub>2</sub>PO<sub>4</sub>, 0.5 g MgSO<sub>4</sub>, 0.2 g KNO<sub>3</sub>, 0.2 mg FeSO<sub>4</sub>·7H<sub>2</sub>O.

0.2 mg ZnCl<sub>2</sub>, 0.2 mg CuSO<sub>4</sub>, 5H<sub>2</sub>O, 0.1 mg MnSO<sub>4</sub>, 4H<sub>2</sub>O, 10 g glucose, 40 g yeast extract in 1 L water (Mallika *et al.*, 1980), was used for growth of *A. flavus* and oxidation of tannin. Tannin (0.1%) was added to the medium separately under aseptic conditions.

The medium was inoculated with a seven day old spore suspension of *A. flavus* in sterile water and the flasks were incubated at room temperature (28-32°C) on a rotary shaker. Controls were carried out side by side in an identical manner but without the mold *A. flavus*.

After 24 h of incubation the media were separated by filtration and the phenolic compounds were estimated before and after polyamide treatment as described above.

*Determination of molecular weight by gel filtration:* The approximate molecular weight of the tannins and the polymerised product in the culture medium was determined by the method of Gelotte (1960) using sephadex G-25 column, catechin ( $M_r$  290), rutin ( $M_r$  160) and tannic acid ( $M_r$  1701) were used as standards. The aromatic compounds were monitored by measuring the absorbance at 280 nm. From the plot of logarithm of molecular weight versus elution volume, the molecular weights of tannin and polymerized product formed in the culture filtrate of mold *A. flavus* were determined (figure 1).



**Figure 1.** Molecular weight of tannin polymer by gel filtration on Sephadex G-25. Samples of 5 mg in 5 ml water or 5 ml culture filtrate of *A. flavus* were subjected to Sephadex G-25 gel filtration chromatography. 5 ml fractions were collected at a flow rate of 15 ml/h.

## Results and discussion

### *Oxidation of tannin by A. flavus*

As mentioned earlier, tannin (0.1%) was added to the growing culture of *A. flavus*, and the amount of tannin oxidised in the medium at various incubation periods by

*A. flavus* was estimated. The extent of oxidation of tannin was calculated by subtracting the value of total phenolic OH groups in the test solution from that of control (without the organism). The results are shown in table 3.

It is known that vegetable tannin can be precipitated by many chemical reagents and these precipitation techniques have become the tool for the estimation of tannin in any of the vegetable tan liquors or in tannery effluents. It can be seen from table 1 that recovery of tannin to nearly 60% could be achieved by precipitation

Table 1. Estimation of tannin by precipitation methods.

Tannin used (mg/ml)	Percentage recovery of tannin		
	Cinchonine sulphate (0.02%)	Caffeine (0.12%)	Lead acetate (0.06%)
0.10	60.0	40.0	58.0
0.25	50.0	40.0	50.0
0.50	45.5	25.0	44.0
1.00	33.3	22.5	32.0
1.50	32.0	20.0	32.0
2.00	32.0	17.5	27.2
2.50	32.0	8.0	22.0

with both cinchonine sulphate and lead acetate and to about 40% by the addition of caffeine. Recovery of tannin by these precipitation methods decreases appreciably when higher concentrations of tannins are present. Hence, these methods cannot be used for the estimation of tannins, although Peri and Pompi (1971) reported a method of estimating the total phenolics after precipitating simple phenolics, non-tanning flavans, condensed and hydrolysable tannins by precipitating with cinchonine sulphate. In the case of lead acetate precipitation, the separation of precipitated lead salts from other materials was very difficult. Hence, lead acetate precipitation method also cannot be adopted for the estimation of tannins. Suntharkar and Jatkari (1938) reported that precipitation with lead acetate did not separate tannins from non-tannins of myrobalan extracts.

When polyamide was used for the adsorption of tannins, it was observed that 0.5 g of polyamide could absorb about 2.5 mg tannins. However, water and 10% methanol eluted only a very insignificant amount of tannin from the column (table 2). Even

Table 2. Specificity of polyamide for adsorption of tannin.

Methanol in water for elution (% v/v)	Retention of adsorbed tannin (%)
0	98.90
10	98.90
30	97.30
60	96.10
90	95.00

when 90% methanol in water was used for elution about 95% of tannin was retained on the column. Thus, polyamide column is ideally suited for the estimation of tannins than any of the precipitation methods where the recovery of tannin was only 32% in cinchonine sulphate, 8% in caffeine and 22% in lead acetate precipitation.

When a mixture of tannins and nontannin phenolics was passed through the polyamide column, almost 99% of all non-tannin phenolics were not retained in the column whereas the tannin was adsorbed by polyamide. The removal of polyphenolic substances like flavonols by the use of polyacaprolactum powder has been demonstrated by Sanderson (1964).

**Table 3.** Oxidation of tannin by *A. flavus*.

Period of oxidation (h)	Tannin oxidised (%)
24	52.9
48	85.3
72	85.3
96	85.3

Having established the reliability of the polyamide adsorption method, it was used to study the oxidation of tannin by the *A. flavus*. After incubating the tannin extract with *A. flavus* and the oxidised product was passed through the polyamide column, all of it was retained on the column. It can be seen from table 3 that about 53% tannin was oxidised by *A. flavus* in 24 h. After a period of 48 h, the oxidation of tannin was maximum (85%). Addition incubation period had no effect on oxidation of the tannin.

The fact that all the reaction product was adsorbed on to the polyamide column indicated that tannin was not degraded to a simpler phenolic compound by *A. flavus*, but instead, a higher mol. wt. compound may have been formed. The determination of the molecular weight of the product by Sephadex G-25 gel filtration, only two compounds, one polymerised product corresponding to  $M_r$  of 4600 and the other unreacted tannin corresponding to a  $M_r$  of 1200 (figure 1) which is the mol. wt. of pure tannin from wattle extract were obtained. Polymerisation of tannins reduces the number of phenolic hydroxyl groups due to the formation of quinone (Swam and Hills, 1959; Smit *et al.*, 1955; Kursenov and Zeprometov, 1949). Smit *et al.*, 1955; Kursenov and Zeprometov, 1949).

Polymerisation of tannin by the mold *A. flavus* may have occurred through quinone formation, as indicated by the maximum absorption of the polymerised product at 420 nm. Thus it is evident from these studies that *A. flavus* could polymerise tannin to a compound of higher molecular weight.

#### Acknowledgements

We are grateful to Dr N. Ramanathan, Acting Director, for permission to publish the paper and to CSIR, New Delhi for the financial assistance to one of the authors (MM).

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## Preliminary studies on the toxicity and mutagenicity of 1-amino-2-naphthol-4-sulphonic acid in *Drosophila melanogaster*

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MS received 27 August 1979; revised 4 February 1980

**Abstract.** The toxicity and mutagenicity of 1-amino-2-naphthol-4-sulphonic acid were analysed in *Drosophila melanogaster*. Rate of development and viability were the two parameters employed to study the toxicity. The frequency of dominant lethals was scored to evaluate the mutagenic effect of the chemical on male and female germ cells. Concentrations of 250 mg and above/100 ml wheat cream agar medium were found to be significantly toxic. Significant number of dominant lethals was induced even by a concentration as low as 50 mg/100 ml medium. Male germ cells were more sensitive than female germ cells.

**Keywords.** 1-Amino-2-naphthol-4-sulphonic acid; *Drosophila melanogaster*; rate of development; viability; dominant lethals.

### Introduction

Sulphonic acids are used in organic synthesis and in the manufacture of dyes and synthetic drugs (Hackh, 1930). Dye intermediates are prominent among the sulphonates commonly found in the market and they include many sulphonic acids. Many of the benzene sulphonic acids are used in the manufacture of synthetic detergents (Richardson, 1957). With such a wide use in industry, it is of importance to analyse the effect of sulphonic acids on biological systems to ensure the safety of occupational exposure of human beings. Several esters of sulphonic acids possess insecticidal, acaricidal, ovicidal, fungicidal and herbicidal properties (Konecny and Demecko, 1973). The fungicidal effects of organic salts of aryl sulphonic acids have also been studies by El-Nawawy *et al.* (1973). Some aromatic sulphonic acids are also known to possess antiviral activity (Akerfeldt *et al.*, 1971). Present investigations report the preliminary studies on the toxic and mutagenic effects of a dye-stuff intermediate 1-amino-2-naphthol-4-sulphonic acid on *Drosophila melanogaster*.

### Materials and methods

#### Toxicity studies

For toxicity studies, 1-amino-2-naphthol-4-sulphonic acid in concentrations of 250, 300, 350 and 400 mg was dissolved in 2 ml alcohol and was thoroughly mixed in 100 ml wheat cream agar medium and these media were poured into vials (3" × 1"). Normal (control I) and 2 ml alcohol supplemented media (control II) served as controls. *D. melanogaster* Oregon K eggs collected by Delcour's technique (1969) were transferred into these vials (25 eggs/vial) so that throughout the development, the larvae were exposed to treated and control foods. Twenty replicates were

maintained for each of the controls and treated media. The census of the emerged flies along with their sexes was taken every day from first to the last day of emergence. From this data, the pattern of emergence as well as the mean development time of the whole group and of the two sexes in each of the chemical concentrations and controls were calculated.

### *Induction of dominant lethals*

Tests for the induction of dominant lethals in male and female germ cells after larval feeding of 1-amino-2-naphthol-4-sulphonic acid were carried out according to the methods of Sankaranarayanan (1967). Since all the concentrations upto 250 mg were found to be lethal, sub-lethal concentrations of 200, 100 and 50 mg were employed to screen for mutagenicity. All the experiments were carried out at a constant temperature of  $24 \pm 1^\circ\text{C}$ .

## Results

### *Pattern of emergence*

Pattern of emergence of flies in controls and the treated series is shown in figure 1. It is clear from the figure that, in the treated series there is a developmental delay. In

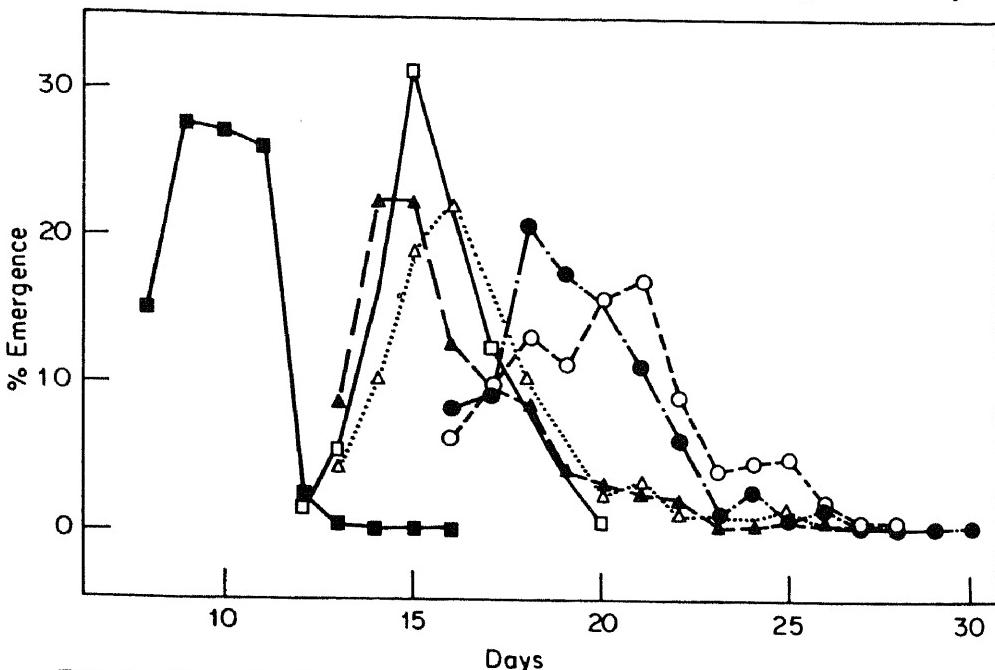


Figure 1. Pattern of emergence of *D. melanogaster* flies in controls and in different concentrations of 1-amino-2-naphthol-4-sulphonic acid.

■—■ control; □—□ alcohol control; Δ—Δ 250 mg; ▲—▲ 300 mg; O—O 350 mg; ●—● 400 mg.

the control I, emergence started on the 9th day and ended on the 17th day. In control II eclosion which started on the 12th day terminated by the 20th day; whereas in 250 mg concentration, emergence commenced on the 13th day extending upto the 27th day. In the highest concentration of 400 mg tested, emergence started on the 16th day and continued upto the 30th day.

### Rate of development

Mean development time for different groups and for the two sexes in each group are presented in table 1. Mean development time for the control I is  $9.83 \pm 0.03$  days and in the control II it is  $15.54 \pm 0.06$  days. In the lowest concentration of 250 mg tested, it is  $16.77 \pm 0.13$  days, while in 400 mg concentration it is  $19.14 \pm 0.21$  days.

**Table 1.** Mean development time of *D. melanogaster*.

Treatment	Mean development time		
	For group	For males	For females
Control	$9.83 \pm 0.03$	$10.02 \pm 0.06$	$9.73 \pm 0.06$
Alcohol (2 ml)	$15.54 \pm 0.06^*$	$15.44 \pm 0.09$	$15.46 \pm 0.15$
1-Amino-2-naphthol-4-sulphonic acid			
250 mg	$16.77 \pm 0.13^*$	$16.89 \pm 0.12$	$16.94 \pm 0.21$
300 mg	$16.08 \pm 0.14^*$	$16.56 \pm 0.08$	$16.21 \pm 0.19$
350 mg	$20.18 \pm 0.15^*$	$20.34 \pm 0.15$	$20.19 \pm 0.24$
400 mg	$19.14 \pm 0.21^*$	$20.29 \pm 0.32$	$19.81 \pm 0.07$

\* Control vs treatment significant at 5% level.

### Viability

Table 2 gives the viability as affected by the chemical. In control I and II lethality is 7.2% and 8.4% respectively, whereas in different concentrations of the chemical, there is a dose-dependent increase in lethality. Thus in 250 mg it is 28.2%, in 300 mg it is 30.0% whereas in 350 and 400 mg it is 39.4% and 50.2% respectively. The number of males and females emerged in each group is also given in table 2.

Many brown and shrunken larvae were found on the sides of the vials with chemical supplemented media. Developmental defects in the form of upheld wings were noticed at all concentrations of the chemical tested (0.5%).

**Table 2.** Viability of *D. melanogaster*

Treatment	No. of adults emerged out of 500 eggs		Mean No. of offsprings/vial	Lethality (%)	Corrected lethality (%)
	Males	Females			
Control	227	237	$23.2 \pm 0.22$	7.2	—
Alcohol (2 ml)	215	247	$22.9 \pm 0.22$	8.4	1.29
1-Amino-2-naphthol-4-sulphonic acid					
250 mg	180	179	$17.95 \pm 0.22$	28.2*	22.63
300 mg	165	185	$17.50 \pm 0.22$	30.0	24.57
350 mg	172**	131**	$15.50 \pm 0.22$	39.4*	34.7
400 mg	141**	108**	$12.45 \pm 0.23$	50.2*	46.34

\* Control vs treatment, significant by analysis variance ( $P < 0.05$ ).

\*\* Sex ratio significant at 5% level.

*Dominant lethals*

Table 3 shows the data on the frequency of dominant lethals in controls and in the treated flies. In control I the percentage of dominant lethals is 3.69%, in control II the percentage of dominant lethals in males is 3.62% and in the females it is 3.75%. While in the males treated with 50 mg of the chemical, it is 12.55% and in the females 8.13%. In the highest concentration (200 mg) of the chemical screened for dominant lethals, it is 20.21% for the treated males and 14.68% for the treated females.

**Table 3.** Frequency of dominant lethals

Treatment	No. of eggs counted	No. unhatched	% Dominant lethals
Control	3445	127	3.686
Alcohol (2 ml)			
Treated males	2811	102	3.628
Treated females	3410	128	3.754
1-Amino-2-naphthol-4-sulphonic acid			
50 mg			
Treated males	2916	366	12.55*
Treated females	2880	234	8.13*
100 mg			
Treated males	3222	638	19.80*
Treated females	2660	340	12.78*
200 mg			
Treated males	3998	808	20.21*
Treated females	3976	584	14.69*

\*  $P < 0.01$ **Discussion**

Survival value and rate of development represent two parameters for evaluating the toxicity (Luning, 1966). Bonnier (1960) has demonstrated that change in the rate of development is due to the compound effects of the genotype and environment. Taking these points into consideration, in the present experiment, the space, the amount of food, the temperature and the number of eggs per vial are identical to that in the controls, and therefore the difference in the rate of development and viability must be due to the chemical and the differences in these parameters are due to the different concentrations used. A significant lengthening of development time is evident in all the concentrations of the chemical tested ( $P < 0.05$ , table 1). But, the delay in development cannot be entirely attributed to the effect of 1-amino-2-naphthol-4-sulphonic acid, because a significant delay is noticed in the alcohol-supplemented control (control II) too. Compared to control II, a significant delay in development is noticed in 350 and 400 mg supplemented diets ( $P < 0.05$ ). However, no significant variation in development time has been observed between the two sexes in each treatment (table 1).

Data in table 2 indicate that 1-amino-2-naphthol-4-sulphonic acid reduces viability in *D. melanogaster*. Analysis of variance has shown that the chemical has significant effects on viability at all the concentrations tested ( $P < 0.05$ ). Table 2 clearly indicates that there is a linear relationship between the degrees of lethality and the concentration of the chemical employed. Developmental defects were also encountered in the form of upheld wings in the chemical-treated flies.

So, from the foregoing discussion it is seen that 1-amino-2-naphthol-4-sulphonic acid is toxic to *Drosophila*. Many derivatives of aromatic sulphonic acids are also shown to have low or moderate toxicity in mice (Kurnatowska and Kurnatowski, 1972). Some of the compounds when injected subcutaneously caused simple fatty degeneration of liver cells, slight proliferation of the white pulp in the spleen and necrosis of a few epithelial cells of the neural tubules. But, the experiments of Gaunt *et al.* (1974) have shown that the food colouring agent Sunset Yellow FCF (disodium salt of 1-R-sulpho-phenyl azo-2-naphthol-6-sulphonic acid) does not exert any long term toxic effect on mice. No evidence for teratogenic or carcinogenic effects of the above chemical was noted by them.

$\chi^2$  homogeneity test for the viability of males and females in each treatment has shown that, at concentrations of 350 and 400 mg, the sex ratio is significantly altered ( $P < 0.05$ , table 2). The viability of females is more affected than those of males thus indicating that the females are more susceptible to the toxic effects of the chemical than males. An alteration in sex ratio due to the effect of chemical in *Drosophila* was reported with Ceresan (Rajasekarasetty *et al.*, 1979; Gayathri and Krishnamurthy, 1979).

Third instar larvae are the most sensitive to the chemical treatment, since they crawl to the sides of the vials, become shrunken and brown, fail to pupate and die. Studies with the colour additive amarnath (a trisodium salt of 1-(4-sulpho-1-naphthyl-azo)-2-naphthol-3, 6-disulphonic acid) is embryotoxic as well foetolethal even in low concentrations (cf. Arnald *et al.* 1976). Another naphthalene derivative 2, 4-dichloro-1-naphthol is found to affect both rate of development and viability in *D. melanogaster* (Krishnamurthy and Vijayan, 1979). Further, based on the findings of Martin and Grossmann (1972a, b) that rufianic acid (1, 4-dioxyanthraquinone-sulphonic acid) has an inhibitory effect on enzyme systems in *Rhizoctonia solani*, a similar enzyme inhibition may probably be operative in causing toxicity in the system in the present study. It can also be recalled that many of the polycyclic hydrocarbons are known to intercalate into DNA and are said to be converted into epoxides by microsomal enzyme systems (Ames *et al.*, 1972).

A dominant lethal mutation by definition is the one which kills the organism when present in a single dose (Auerbach, 1962). Table 3 reveals that, 1-amino-2-naphthol-4-sulphonic acid is significantly mutagenic in male and female germ cells at all concentrations tested ( $P < 0.01$ ). But, a higher frequency is noticed in the male germ cells than the female germ cells at all concentrations tested. The experiments of Arnald *et al.* (1976) indicate that the colour additive amaranth has no significant effect on the frequency of dominant lethal mutations in male mice. Mono-, di-, and trisulphonic acids are also known to produce a reversible inhibition of sulphate equilibrium exchange in human red cells (Zaki *et al.*, 1975). The findings of Misra *et al.* (1975) indicate a neuroexcitatory and neurotoxic effects of sulphonic acids. In the light of all this, the effects of sulphonic acids on biological systems need careful monitoring.

### Acknowledgements

The authors are grateful to Prof. M. R. Rajasekarasetty for his constructive criticism and constant encouragement. One of the authors (MVG) is grateful to University Grants Commission, New Delhi for financial assistance.

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## **Immunoprecipitation of 70S, 50S and 30S ribosomes of *Escherichia coli***

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MS received 4 December 1979

**Abstract.** Antibodies were raised in rabbits against 70S ribosomes, 50S and 30S ribosomal subunits individually. Purified immunoglobulins from the antiserum against each of the above ribosomal entities were tested for their capabilities of precipitating 70S, 50S and 30S ribosomes. The observations revealed the following: (i) The antiserum (IgG) raised against 70S ribosomes precipitates 70S ribosomes completely, while partial precipitation is seen with the subunits, the extent of precipitation being more with the 50S subunits than with 30S subunits; addition of 50S subunits to the 30S subunits facilitates the precipitation of 30S subunits by the antibody against 70S ribosomes. (ii) Antiserum against 50S subunits has the ability to immunoprecipitate both 50S and 70S ribosomes to an equal extent. (iii) Antiserum against 30S subunits also has the property of precipitating both 30S and 70S ribosomes. The differences in the structural organisation of the two subunits may account for the differences in their immunoprecipitability.

**Keywords.** Ribosomes; ribosomal subunits; antibody; immunoprecipitability.

### **Introduction**

The topography of ribosomal proteins has been investigated by a number of elegant experimental approaches including immunology coupled with electron microscopy (for review, see Stoffler and Wittmann, 1977). The availability of specific antibodies to each of the 54 ribosomal proteins from *Escherichia coli* ribosome was a prerequisite for these studies. Apart from antisera to two proteins pairs L7 and L12 as well as S20 and L26), no immunological cross-reaction has been found between the different antisera raised individually against all the proteins of the ribosome. Some immunological approaches towards the study of the structure of the ribosome have been made in this laboratory (Das and Burma, 1979). During these preliminary investigations antiserum was raised in rabbits against intact *E. coli* 70S ribosome and it was observed that such antiserum efficiently precipitates 70S as well as 50S ribosomes but not 30S ribosomes. Subsequently antisera were raised against individual ribosomal subunits and detailed studies on the immunoprecipitation of the two subunits were carried out. These studies will be presented here. Similar observations have been made with rat liver ribosome (Stoffler *et al.*, 1978).

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### Materials and methods

The sources of the materials used have been mentioned in an earlier publication (Das and Burma, 1979). Sulphur-35 [ $^{35}\text{S}$ ] as sulphuric acid (carrier-free) in dilute HCl was the product of Bhabha Atomic Research Centre, Trombay, Bombay.

The immunisation of rabbits, isolation of immunoglobulin (IgG) from antiserum and methods of immunoprecipitation etc. have already been described (Das and Burma, 1979).

#### *Preparations of [ $^{35}\text{S}$ ]-labelled ribosome and its subunits*

The method described by Sun *et al.* (1974) was followed for growing *E. coli* in [ $^{35}\text{SO}_4^{2-}$ ]-containing medium. Under this condition, the cells incorporated 60-70% of the radioactivity. These radioactive cells (2 g wet weight) were mixed with nonradioactive cells (5 g) for preparing [ $^{35}\text{S}$ ]-labelled ribosomes. Ribosomes were prepared as described earlier (Datta and Burma, 1972). The final ribosomal preparation had a specific activity of  $8.2 \times 10^4$  counts/min/A<sub>260</sub> unit. The 50S and 30S subunits were isolated from [ $^{35}\text{S}$ ]-labelled ribosomes following the method described by Godson and Cos (1970). Final preparations of 30S and 50S subunits had specific activities of  $7.3 \times 10^4$  and  $5.1 \times 10^4$  counts/min/A<sub>20</sub> unit, respectively.

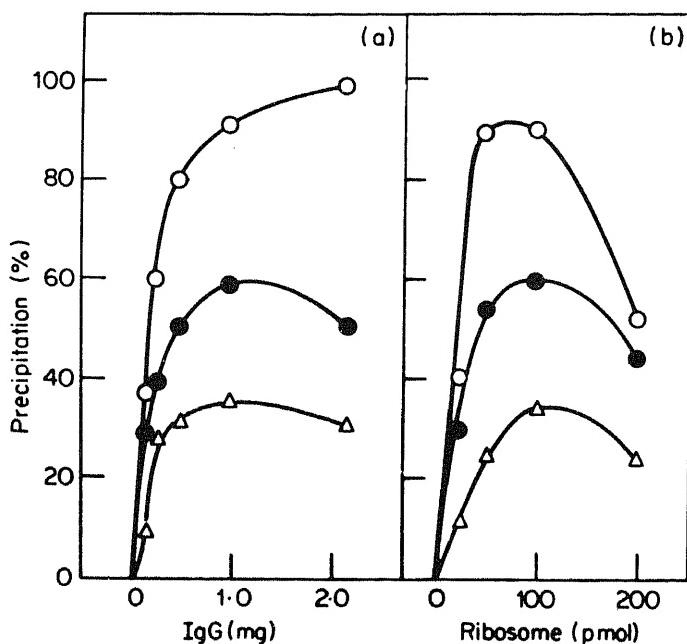
#### *Immunoprecipitations of radioactive ribosomes by varying amounts of IgG or antisera*

The method of Chu *et al.* (1976) was used with some alterations. [ $^{35}\text{S}$ ]-labelled 70S, 50S and 30S ribosomes (200  $\mu\text{g}$  containing  $2.3 \times 10^4$  counts/min) were individually mixed with increasing amount of IgG or antisera, as the case may be, in presence of 0.05 M Tris-HCl, pH 7.4, 0.01 M magnesium-acetate and 0.5 M NH<sub>4</sub> Cl in a total volume of 0.5 ml and the mixture and incubated at 37°C for 30 min and subsequently at 0°C for 1 h. The precipitate obtained by centrifugation at 15,000 g for 15 min at 0°C was washed twice with 0.05 M Tris-HCl, pH 7.4, 0.5 M NH<sub>4</sub> Cl and 1% (v/v) Triton X-100 (1 ml) and finally suspended in 4M urea containing 50% acetic acid and 1% Triton X-100 (0.25 ml). Aliquots (0.1 ml) were added to 5 ml of Bray's solution (Bray, 1960) and counted in a Nuclear Chicago liquid scintillation counter. Percentage of ribosomes precipitated was calculated from the radioactivity in the immunoprecipitate.

### Results

#### *Immunoprecipitation of [ $^{35}\text{S}$ ]-labelled 70S ribosome and its subunits by IgG isolated from the antiserum raised against 70S ribosome*

It is evident from the results presented in figure 1a that there is more immunoprecipitate formed with the increasing amount of IgG used for a fixed amount (200  $\mu\text{g}$ ) of 70S or 50S or 30S ribosomes. However, a wide difference is found between the extents of precipitation of ribosomes and their subunits. For example, 90 to 100% of the 70S ribosome is precipitated by the addition of 1 to 2 mg of IgG. In case of 30S ribosome, however, only one third or less amount is precipitated by the addition of similar amount of IgG; actually 0.25 mg IgG is sufficient for the purpose. In case of 50S ribosome about 60% of the subunit is precipitated with the addition of 1 mg of IgG. Doubling the amount of IgG leads to a somewhat less precipitation (50%).



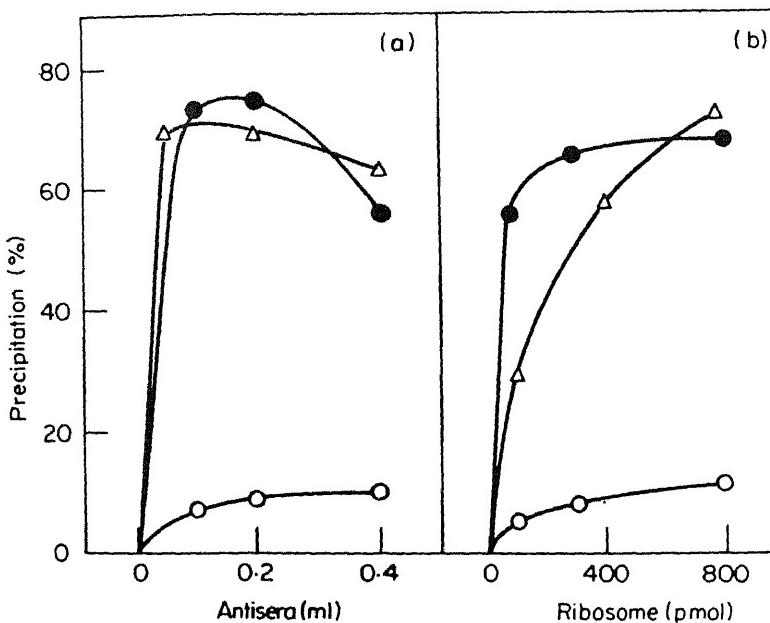
**Figure 1.** Immunoprecipitation of [<sup>35</sup>S]-labelled 70S ribosome and its subunits by IgG isolated from the serum raised against 70S ribosome. [<sup>35</sup>S]-labelled 70S, 50S and 30S ribosomes (200 µg, 2.5 × 10<sup>4</sup> counts/min) were incubated with different amounts of IgG(A) and a fixed amount of IgG (0.75 mg) was incubated with different amounts of [<sup>35</sup>S]-labelled 70S, 50S and 30S ribosomes (B). The percentage of precipitation was determined from the radioactivity of the precipitate formed. The details have been described under Materials and methods. —Δ— 30S; —●— 50S; —○— 70S.

Similar observation is made when a fixed amount of IgG (0.75 mg) was added to varying amounts of ribosomes (figure 1b). Under this condition 50–100 pmol of ribosomes are precipitated to the extent of 90%. However, when excess ribosome is added there is much less precipitation, as expected. Under the same condition, however, only 60% of 50S ribosomes is precipitated. There is a slightly less amount of precipitation with the addition of increasing amount of the same subunit. Again the precipitation of the 30S subunit is comparatively much less and only 30–35% of the 30S subunit is precipitated under identical conditions. These results clearly indicate that the antibody raised against 70S ribosome behaves differently towards 50S and 30S ribosomes so far as the extent of precipitation is concerned.

#### *Immunoprecipitation of 70S ribosome and its subunits by varying amounts of antisera raised against 50S ribosome*

The immunoprecipitating capacity of antibodies raised separately against the individual subunits was tested against 70S ribosome and its subunits. Since it was known earlier that in the case of 70S ribosome, the behaviour of the antiserum and that of IgG prepared from the antiserum are practically the same, (data not shown), the antiserum was used directly for precipitation of ribosomes in these experiments. The results obtained with antisera raised against 50S ribosome are shown in figure 2.

When a fixed amount (200 µg) of ribosome is used, both 70S and 50S ribosomes are precipitated to the maximum extents (70–75%) by 0.05 to 0.10 ml of the antisera

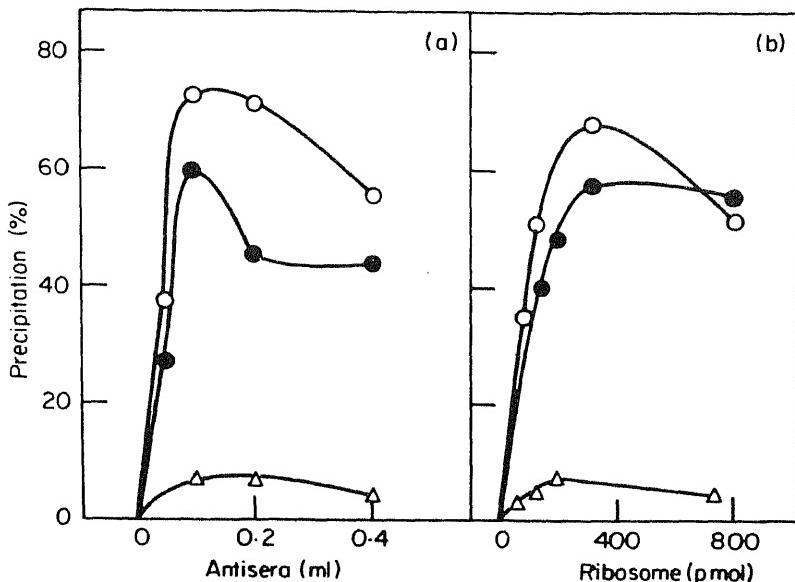


**Figure 2.** Immunoprecipitation of [<sup>35</sup>S]-labelled 70S ribosome and its subunits by antiserum raised against 50S ribosome. [<sup>35</sup>S]-labelled 70S, 50S and ribosomes (200 µg, 2.5 × 10<sup>4</sup> counts/min) were incubated with different amounts of antisera (A) and a fixed amount of antisera (0.1 ml, 7 mg) was incubated with different amounts of [<sup>35</sup>S]-labelled 70S, 50S, and 30S ribosome (B). The per cent precipitation was calculated from the radioactivity in the immunoprecipitate as described under Materials and methods. —○— 30S; —△— 50S; —●— 70S

(figure 2b). Increasing the amount of antisera does not lead to any further increase in the immunoprecipitate. On the other hand, there is slightly less amount of precipitate formed. However, there is small amount of precipitate formed with the 30S ribosome. Since the 50S ribosome used for immunisation had slight contamination of 30S ribosome (and vice versa), this was not quite unexpected. Similar observation was made when a fixed amount of antiserum (0.1 ml) and varying amount of ribosomes were used (figure 2b). This amount of antiserum is capable of precipitating 70% of either 70S or 50S ribosome when 800 pmol of either ribosomes are used. With the use of lesser amount of ribosome, lower amount of ribosome is precipitated, as expected. Again as in the earlier case, 5-10% of 30S ribosome is precipitated by the antiserum raised against the 50S ribosome. This may be due to the cross-contamination of the preparations, as discussed above.

#### *Immunoprecipitation of [<sup>35</sup>S]-labelled 70S ribosome and its subunits by antiserum raised against 30S ribosome*

The results obtained with varying amounts of antiserum raised against 30S ribosome have been presented in figure 3a and those obtained with varying amounts of ribosomes have been presented in figure 3b. There is increasing amount of immunoprecipitation of 70S and 30S ribosomes with the increasing amount of antiserum used. With the addition of 0.1 ml of the antiserum to 200 µg of 70S ribosome there is about 70-75% precipitation. However, on addition of double this amount, somewhat less amount



**Figure 3.** Immunoprecipitation of [ $^{35}$ S]-labelled 70S ribosome and its subunits by antiserum raised against 30S ribosomes. Incubations were done as described in the legend to figure 2 with the difference that the antiserum raised against 30S ribosome was used. The details have been mentioned in Materials and methods. —○— 70S ; —●— 30S ; —Δ— 50S

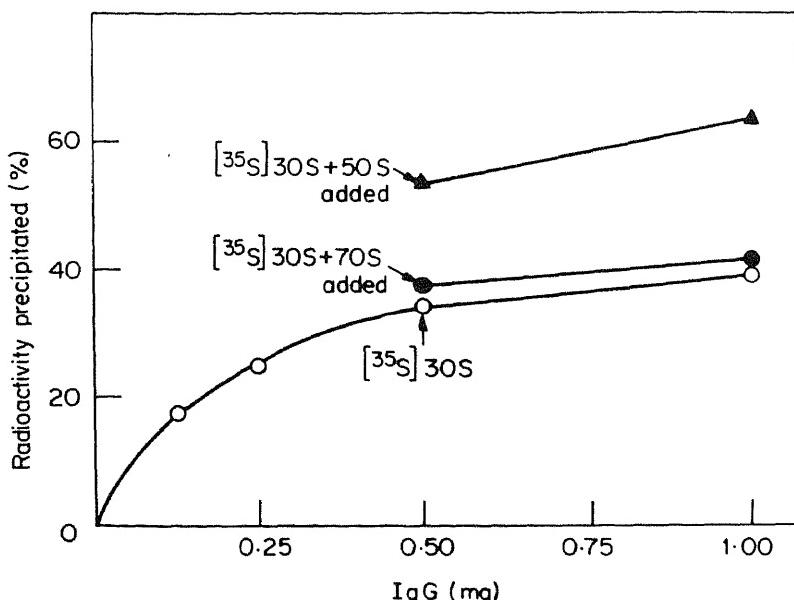
of precipitate is formed. With the 30S ribosome, maximum precipitation (60%) was obtained with 0.1 ml of antiserum. With increasing amounts of antiserum lesser quantity of precipitate (40-45%) is formed. Again there is small but significant amount of immunoprecipitation of the 50S ribosome, possibly due to cross-contamination. Similar results were obtained when a fixed amount (0.1 ml) of antiserum and varying amount of ribosomes were used (figure 3a). Maximum precipitation obtained in case of 70S and 30S ribosomes is 55-65%.

#### *Effect of 50S ribosome on the immunoprecipitation of 30S ribosome with IgG against 70S ribosome*

It is clear from the results presented in figure 1 that the 30S ribosome is not as efficiently precipitated as the 50S ribosome. In order to check whether the 50S ribosome will help in the precipitation of 30S ribosome, non-radioactive 70S ribosome or 50S subunits were added and further incubated. It is clear from the results presented in figure 4 that the addition of unlabelled 70S ribosome does not help in the precipitation of 30S subunit by two different concentrations of IgG against 70S ribosomes. However, addition of unlabelled 50S subunit enhances the precipitation of 30S subunit to a considerable extent.

#### **Discussion**

It is clear from the results presented above that the 50S ribosome of *E. coli* is more efficiently precipitated than the 30S ribosome by the antisera raised against either intact 70S ribosome or individual subunits. As mentioned in the introduction, this difference in behaviour of the two subunits has also been observed in case of rat



**Figure 4.** Effect of addition of unlabelled 70S and 50S ribosomes on the immunoprecipitation of [<sup>35</sup>S]-labelled 30S-ribosome by IgG isolated from the antiserum raised against 70S ribosome. The immunoprecipitation of [<sup>35</sup>S]-labelled 30S ribosomes by IgG was carried out as described in the legend to figure 1. Same amount of nonradioactive 70S ribosome or 50S ribosome was added at the point indicated by the arrows.

liver ribosomes, although no explanation has been provided (Stoffler *et al.*, 1978). One of the reasons in case of *E. coli* ribosomes could be that the proteins of 50S ribosome are more antigenic than those of 30S ribosome but this seems unlikely from the detailed studies carried out by Stoffer and his coworkers (Stoffler *et al.*, 1973; Morrison *et al.*, 1977). It may also be argued that 50S ribosome is double in size than the 30S ribosome hence more efficiently precipitated but still the latter is a giant molecule composed of 21 proteins, therefore the size would hardly matter. One other argument could be that more soluble complex is formed in case of 30S ribosome. However, ultracentrifugation experiments (results not presented) do not corroborate this. On the other hand, it may be speculated that the 30S ribosome has a more compact structure than the 50S ribosome, the better accessibility of the proteins in the larger ribosome helps in its efficient precipitation. The overall structural organisation of the two subunits may also be quite different (Burma, 1979). In many respects the two ribosomal subunits behave differently, for example, 50S ribosome is more susceptible to the action of RNase I than 30S ribosome (Datta and Burma, 1972); however, when a part of the structure of 50S ribosome is removed by the action of RNase I, the 50S core particle behaves like 30S ribosome (Raziuddin *et al.*, 1979). Further, the intercalation of the dye, ethidium bromide into RNA of the 50S ribosome is very much dependent on the concentration of Mg<sup>2+</sup>, it is much less dependent in the case of 30S ribosome (Burma, *et al.* 1979). There is also considerable difference in the action of trypsin on the two subunits, the larger ribosome being more susceptible than the smaller one (Ali, 1978). Such difference is also observed in the action of formaldehyde on the two subunits (Chatterji *et al.*, unpublished observations). Very recently it has been observed that monovalent cations have

tremendous influence on the structure of 50S ribosome whereas the effect is very small in case of 30S ribosome (Raziuddin, unpublished observations). Considering these observations along with the that made in this paper it appears that there is a basic difference in the structural organisation of the two subunits and this may account for the inefficient precipitation of 30S ribosome in comparison with the 50S ribosome.

### Acknowledgements

Sincere thanks are due to the University Grants Commission, New Delhi for financial assistance. The work was initiated in this laboratory by Dr. M. Das, Department of Biochemistry and Biophysics, Kalyani University, Nadia, West Bengal.

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## Chromatic adaptation and photoreversal in blue-green alga *Calothrix clavata* West

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MS received 23 August 1979; revised 19 November 1979

**Abstract.** Complementary chromatic adaptation, a well-established phenomenon in some blue-green algae, has been observed in *Calothrix clavata*, a heterocystous blue-green alga of the family Rivulariaceae. The chromatic adaptation has been observed for fluorescent and incandescent light by measuring the absorption spectra. The material grown in fluorescent light forms more of phycerythrin whereas more of phycocyanin tends to be formed in incandescent light. Besides this, photoreversal was observed by transferring the incandescent light grown alga to fluorescent light conditions and vice-versa. Effect of photoreversal and chromatic adaptation has also been discussed for this alga under different monochromatic light conditions. The influence of different light conditions on morphological changes, heterocysts and hormogonia formation has also been investigated. Both chromatic adaptation and photomorphogenetic phenomena in this alga show the involvement of some photoreversible (red:green) pigment.

**Keywords.** Chromatic adaptation; phycerythrin; phycocyanin; *Calothrix*.

### Introduction

Chromatic adaptation is one of the photomorphogenetic responses known to occur in blue-green algae. This is a phenomenon wherein the relative levels of different biliproteins are controlled by spectral quality of the light in which the algae are grown. These changes in pigment composition are usually due to differences in the relative rates of synthesis and total amounts of biliprotein pigments i.e. phycocyanin, phycerythrin and allophycocyanin. Biliproteins are important accessory photosynthetic pigments and their ability to adapt chromatically confers an obvious ecological advantage in that the organism is able to make maximum photosynthetic use of the available light. This phenomenon has been well studied in a blue-green alga *Tolyphothrix tenuis* (Fujita and Hattori, 1960a, b, 1962; Diakoff and Scheibe, 1973; Ohki and Fujita, 1978). Coupled with the changes at the subcellular level in pigment composition, gross macroscopic photomorphogenetic responses also occur (Bennett and Bogorad, 1973). In this work, we describe the red:green, incandescent: fluorescent light effects on biliprotein synthesis along with some photomorphogenetic changes in the blue-green alga *Calothrix clavata* West.

### Materials and methods

#### Growth conditions

*Calothrix clavata* is a heterocystous blue-green alga. Its stock clonal axenic culture has been maintained on agar slants (1% v/v) at  $28 \pm 2^\circ\text{C}$ , illuminated with 1500 lux of incandescent light. Allen and Arnon's medium (1955) has been used as the basal medium in all experiments.

Phosphate was autoclaved separately and added to cooled sterilised medium aseptically to avoid precipitation. In dark-grown culture experiments, 1% glucose (w/v) supported the growth of the alga.

#### *Light treatments*

The filaments grown in incandescent light were harvested, washed twice with sterilised double distilled water, and homogeneous suspension prepared by repeated shaking with sterilised glass beads. Equal aliquots from this suspension were inoculated in culture tubes and flasks and grown under different light conditions. Before giving short light exposures, the alga is kept in dark for 20 h to exhaust any carry-over effect of light. The intensity of both types of irradiance used i.e. incandescent and fluorescent, was 1500 lux. The filaments were grown in different light conditions for varying periods. After every exposure, 20 h dark incubation was given and then they were harvested for pigment analysis. Monochromatic red and green irradiance were produced by passing incandescent light through standard filters (Carolina Biological Supply Company, USA) and a water column to avoid any heating effect. Rest of the procedure is same as above.

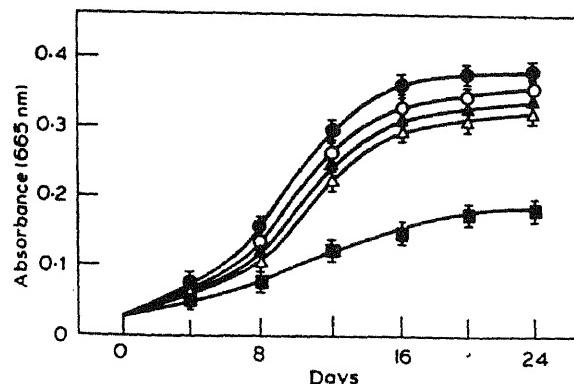
#### *Determination of absorption spectrum*

Pre-illuminated filaments were harvested by centrifugation (3000 rpm for 10 min) and washed with sterilised distilled water. After extraction of the pigments into 90% acetone, the water-soluble pigments, phycoerythrin and phycocyanin were extracted in distilled water by repeated freezing and thawing of the pellet. The absorbance spectrum was determined in a Spectronic-20 spectrophotometer in the wavelength region of 400-700 nm.

For photomorphogenetic studies, the filaments were grown on agar-plates with different light treatments as described above and the changes in their morphology, if any, were observed microscopically.

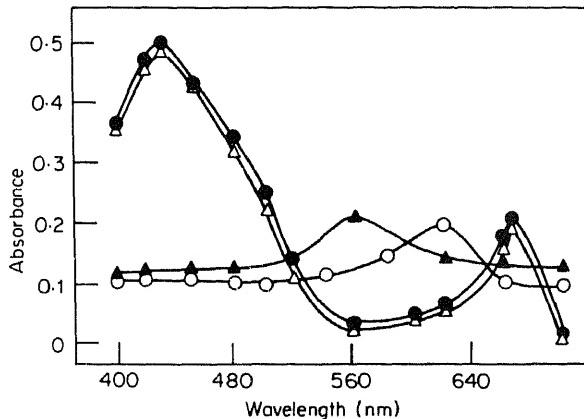
#### **Results**

The growth curves (figure 1) show that glucose (1%) in incandescent light conditions enhanced the growth of this alga. It also supported the growth in the dark but at a



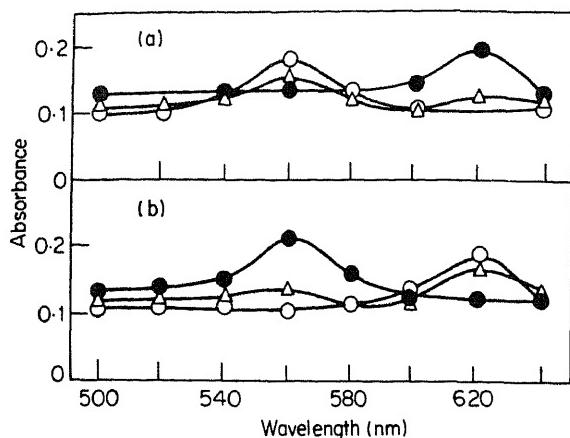
**Figure 1.** Growth of *Calothrix clavata* under different conditions. 1% glucose in dark; ■—■ ; Fluorescent light grown, Δ—Δ ; Incandescent light grown, ▲—▲ ; 20 mM KNO<sub>3</sub> in incandescent light ●—● ; 1% glucose in incandescent light O—O . 10 ml of cell suspension extracted in 10 ml of 90% acetone.

much reduced rate. Potassium nitrate (20 mM) slightly enhanced the growth. The growth in fluorescent light grown culture was approximately the same as that obtained in incandescent light.



**Figure 2.** Absorption spectra of acetone soluble pigments of fluorescent light grown culture  
 $\Delta - \Delta$ ; Incandescent light grown  $\bullet - \bullet$  and water soluble pigments in fluorescent light grown  
 $\blacktriangle - \blacktriangle$ , Incandescent light grown  $O - O$ . Inoculum size same as in figure 1. On 10th day of growth, 10 ml of cell suspension extracted in 10 ml of extracting medium.

The absorption spectra for incandescent and fluorescent light grown cultures are shown in figure 2. The chlorophyll peaks were observed at 425 nm and 665 nm. The bili-proteins showed a characteristic pattern under different light conditions. There was only one peak at 560 nm in fluorescent light-grown culture and the peak shifted to 620 nm in the incandescent light grown cultures of same age and of nearly same chlorophyll content. The peak at 560 nm is due to phycoerythrin (purple pigment) and at 620 nm is due to phycocyanin (blue pigment). Similar results have been found by growing the cultures, supplied with glucose, under red and green lights. The culture could visually be distinguished on the basis of its colour (fluorescent light grown culture was brown in contrast to green under incandescent light conditions). The intermediate stage of reversal of pigment formation in this alga under different growth conditions in terms of light quality was determined (figure 3). When fluorescent light grown alga (9 days old) was transferred to incandescent light for three day, there was emergence of a small peak at 620 nm in addition to one larger peak at 560 nm (but still smaller than the control). Growth for additional 6-8 days in incandescent light spectrum clearly showed a sharp peak at 620 nm but no peak at 560 nm suggesting thereby the photoreversal of phycoerythrin to phycocyanin when light conditions shifted from fluorescent to incandescent (figure 3a). In figure 3b, the same phenomenon was observed but in the reverse direction i.e. when incandescent light grown culture was transferred to fluorescent light conditions, there was photo-reversal of phycocyanin to phycoerythrin. When the incandescent light grown culture was transferred to fluorescent light conditions for three days, there was formation of a small peak at 560 nm in addition to a peak at 620 nm. But after 6-8 days, there was only one peak 560 nm. The same pattern was obtained with red and green



**Figure 3.** Formation of Phycocyanin and Phycocrythrin under different light quality conditions. Inoculum size same as in figure 1. 10 ml of cell suspension extracted in 10 ml of distilled water.

- (a) Water soluble pigments of fluorescent light grown culture transferred to incandescent light after 9 days O—O, 12 days Δ—Δ, 18 days ●—●. (b) Water soluble pigments of incandescent light grown culture transferred to fluorescent light after 9 days o—o, 12 days Δ—Δ, 18 days ●—●.

light spectra and vice-versa. The ratio of phycoerythrin/phycocyanin, in fluorescent light grown culture is more than twice the value of the incandescent light green culture (table 1).

**Table 1.** Phycoerythrin and Phycocyanin ratios under different growth and light conditions.

Light treatment	PE/PC (560 nm/620 nm)			
	AA*	AA+N@	AA+G(L)†	AA+G(D)‡
Incandescent light (control)	0.68	0.66	0.72	0.90
Red light: 1 h	0.68	0.67	0.71	0.89
5 h	0.68	0.67	0.73	0.86
Green light: 1 h	0.78	0.72	0.76	0.92
5 h	0.81	0.78	0.80	0.98
Red:Green 2:2 h	0.80	0.70	0.82	0.90
Green:Red 2:2 h	0.70	0.66	0.74	0.88
Incandescent light (10 days)	0.67	—	—	—
Fluorescent light (10 days)	1.48	—	—	—

\* AA: Allen and Arnon's medium.

@ AA+N: Supplemented with 20 mM potassium nitrate.

† AA+G(L): Supplemented with 1% glucose and grown in light.

‡ AA+G(D): Supplemented with 1% glucose and grown in dark.

PE: Phycoerythrin, PC: Phycocyanin.

With short light exposure (1-5 h), ending with green, phycoerythrin/phycocyanin increases and in red light exposures, this ratio decreases thereby showing that even short exposures to these light qualities affect the biliprotein synthesis (table 1). This ratio was nearly the same for glucose supplemented media and slightly less in nitrate supplemented media, although glucose given in the dark increases this ratio.

The main morphogenetic change is that in red light or incandescent light, the basal heterocysts tend to form pairs or occur as chains of three each although paired heterocysts rarely occur in intercalary positions. In fluorescent or green light, the basal heterocyst is single, just as in control culture, but intercalary paired heterocysts do occur in almost 10% of the filaments. The cell size gradient is almost the same in all treatments but in nitrate supplemented culture this gradient is absent as heterocysts are also absent and the formation of hormogonia is much more than the control. The sheath was nearly absent in such cultures (table 2). In glucose supplemented

**Table 2.** Morphogenetic changes under different conditions.

Treatment	Cell size gradient	Heterocyst position	Hormogonia	Sheath	Granulation	Polar plug	Branching	Colour of algal culture
Incandescent light	++	BPH+IH	50% (10-30C)	P	P	++	1%	Green-colour
Fluorescent light	++	BH+IPH(10%)	10% (8-20C)	P	-	+	-	Brown-colour
Incandescent +20mMKNO <sub>3</sub>	-	-	80% (6-28C)	A	-	-	-	Green
Incandescent +1% glucose	+	BH+IPH(10%) BPH(2%F)	10% (6-20C)	P	-	-	1%	Green
Green light	++	BH+IPH(5%)	3% (6-20C)	P	-	+	-	Green brown
Red light	+++	BPH+IH(Rarely)	15% (8-26C)	P	+	+	0.5%	Green

- Absent; + Poorly present; ++ Present; +++ Distinct.

BH: Basal heterocyst; BPH: Basal Paired heterocyst; IPH: Intercalary paired heterocyst; IH: Intercalary heterocyst; F: Filaments C: Cells.

P: Present

A: Absent.

medium, in incandescent light, it formed basal heterocysts and paired intercalary heterocysts in about 10% of the filaments. Basal heterocysts in a chain of three have been observed in nearly 2% of the filaments. Next to heterocyst, 4-10 cells are quite bigger in size and appear bead shaped. Transfer of cultures to different light qualities often changed their mode of heterocyst formation and hormogonia production specifically.

## Discussion

Various hypotheses have been proposed to explain the photocontrol of chromatic adaptation in blue-green algae (Fujita and Hattori, 1962; Diakoff and Scheibe, 1973; Bogorad, 1975; Björn and Björn, 1976; Tandeau de Marsac, 1977). The hypothesis proposed by Diakoff and Scheibe (1973) and Bogorad (1975) seems most attractive. It suggests that phycoerythrin/phycocyanin formation is controlled by photochemical

activity of a pigment independent of precursors of phycobiliprotein formation. Others suggest that there are precursors for both phycoerythrin and phycocyanin and the quality of light determines which type of biliprotein is to be formed (Fujita and Hattori, 1962).

The green light elicits production of phycoerythrin and red light produces phycocyanin. Similar is the case with fluorescent and incandescent light. In fluorescent light, the percentage of input lamp watts radiated in the green band is more than in the red band (Withrow and Withrow, 1956) whereas it is not the case in incandescent light spectrum, so there is induction in the synthesis phycoerythrin in fluorescent light.

According to Ohki and Fujita (1978), phycoerythrin formation had a 3-5 h lag period and then occurred almost linearly for 15-20 h until the formation slowed down and the pigment content reached a maximum level. We have found a maximum phycoerythrin/phycocyanin ratio of 1.5 and a minimum of 0.6, so that under the specific light conditions only one of these phycobilins predominates. With short exposures, values in between these two extremes were obtained thereby suggesting that there may not be any interconversion among the already formed phycocyanin and phycoerythrin, but only the precursors present at that time may have been induced by some independent pigment responding to the light quality (Diakoff and Scheibe, 1973; Ohki and Fujita, 1978). So there may be some pigment functioning as phycochrome, similar to the phycochrome of higher plants, but with red:green reversal (see Shropshire, 1977). Besides the phycoerythrin induction, other photo-controlled phenomena are known to be involved in the growth of some blue-green algae (Bennett and Bogorad, 1973; Lazaroff, 1973; Diakoff and Scheibe, 1975; Tyagi and Ahluwalia, 1978). The observation of changes in heterocysts and hormogonia formation in the present study supports this hypothesis.

### Acknowledgements

Thanks are due to Council of Scientific and Industrial Research, New Delhi, for the award of fellowships to ASA and RKR.

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## The role of follitropin and lutropin on the ovarian function in rats

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MS received 31 January 1980

**Abstract.** Adult cycling female rats were treated with antisera to highly purified human follitropin and lutropin for eight days. The effect of this treatment on the *in vitro* steroidogenic response of the ovarian cells isolated from these rats to follitropin and lutropin has been investigated. Neutralisation of follitropin did not have significant effect on steroid production in response to lutropin. However, neutralisation of lutropin resulted in a very significant inhibition of response to both follitropin and lutropin.

**Keywords.** Follitropin; lutropin; radioimmunoassay; rat; steroidogenesis

### Introduction

It is well established that lutropin (LH) stimulates steroidogenesis under *in vivo* and *in vitro* conditions in a variety of species (Savard *et al.*, 1965; Armstrong, 1968; Eaton and Hilliard, 1971; Carlson *et al.*, 1971). However, recent studies (Tsafriri *et al.*, 1976; Grimek *et al.*, 1976; Dorrington and Armstrong, 1975; Armstrong and Papkoff, 1976) using highly purified preparations of ovine follitropin (FSH) have shown that FSH by itself can induce ovulation in rats, stimulate follicular adenylate cyclase and steroidogenesis under *in vivo* and *in vitro* conditions. Jagannadha Rao *et al.* (1974) have shown in a detailed study, using antiserum to LH and FSH anti-serum freed of LH antibodies, that FSH has no significant role in induction of ovulation in both rats and hamsters. However, recent studies of Eppig (1979) based on the production of plasminogen activator by granulosa cells, prior to plasmin acting to weaken the follicular wall and hence ovulation, suggests that as far this criterion is concerned, ovulation is more sensitive to FSH than LH. In the present communication the relative role of FSH and LH in stimulation of production of progesterone and estradiol in rat ovarian cells after neutralisation of endogenous FSH or LH by specific antisera to human FSH and LH has been studied.

### Materials and methods

#### *Hormones*

Highly purified human pituitary FSH and LH were prepared and characterised according to published procedure (Sairam *et al.*, 1978). Rat LH and FSH were obtained from the Hormone Distribution Office, NIAMDD, Bethesda, Maryland, USA. The biological activity of FSH was monitored using a specific *in vitro* assay in

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Abbreviations: LH, lutropin; FSH, follitropin; RIA, radioimmunoassay; A/S, antiserum.

this laboratory (Jagannadha Rao and Ramachandran, 1975). LH activity was tested in the *in vitro* Leydig cell assay described earlier (Ramachandran and Sairam, 1975).

#### *Immunisation*

Antisera to human pituitary FSH and LH were raised in New Zealand white male rabbits according to the procedure of Vaitukaitis *et al.* (1971). The antisera were characterised by agar gel diffusion and quantitative precipitin tests. The ability of the antisera to neutralise the biological activities of rat FSH and LH was ascertained by testing the capacity of the antisera to inhibit the response to rat FSH and LH in the *in vitro* bioassays mentioned above.

#### *Animals and other procedures*

Sprague-Dawley female rats (160-180 g) were obtained from Simonsen Laboratories, Gilroy, CA, USA. Rats were used after observing three consecutive estrous cycles. To groups of five rats, each 0.5 ml of normal rabbit serum or antiserum to FSH or LH was administered for a period of eight days by subcutaneous route. Twenty-four hours after the last injection, rats were anaesthetised by nembutal, blood collected by cardiac puncture, serum separated and stored at -20°C until further processing.

#### *Preparation of ovarian cells*

The ovaries and uteri were dissected free of connective tissue and weighed in a torsion balance. From each group the ovaries were pooled, minced and taken in 30 ml of Krebs Ringer bicarbonate buffer pH 7.4 containing 2 mg bovine serum albumin, 1 mg glucose and 0.01 mg Lima Bean Trypsin Inhibitor per ml (incubation buffer). After one wash with the buffer, the minced tissue was digested in the same buffer with 5 mg collagenase (Worthington) per ml (10 ml) for 1 h at 37°C with gentle shaking. After digestion, the suspension was allowed to settle and the supernatant removed and discarded. Thirty ml of fresh incubation buffer was added to each tube and the suspension was dispersed by draining in and out of a plastic syringe with a tygon tubing at the end. The suspension was allowed to settle and the supernatant removed and filtered through two layers of cheese cloth. The process was repeated three times and filtered supernatants pooled and centrifuged gently at room temperature for 15 min at 500 g. The cell pellet was washed twice with the incubation buffer and resuspended in a known volume of the incubation buffer. A known aliquot of the suspension was diluted with sterile 0.9% saline and the cell number was determined in a coulter counter.

#### *Incubation*

From each group, cells ranging from  $4 \times 10^5$  to  $9 \times 10^5$ /tube were incubated in a plastic tube (Falcon) in a total volume of 0.5 ml with or without FSH or LH in an atmosphere of 95% O<sub>2</sub> and 5% CO<sub>2</sub> for 2 h at 37°C. At the end of incubation the tubes were stored at -20°C until further processing.

#### *Steroid estimation*

Progesterone and estradiol -17 $\beta$  were estimated by radioimmunoassays standardised in this laboratory. Progesterone and estradiol -17 $\beta$  bovine serum albumin conjugates were prepared according to the procedure of Erlanger *et al.* (1975). Antibodies were raised in male rabbits and characterised by checking the cross

reactivity with related steroids, namely pregnenolone, testosterone, dehydro-epiandrosterone, aldosterone and corticosterone; it was found to be less than 0.001%. Serum was thawed and extracted with 3 volumes of diethyl ether and ether layer concentrated and reconstituted in 0.01 M phosphate buffer. Recovery was monitored by addition of [<sup>3</sup>H]-steroids (2000 cpm) and was found to be greater than 80%. Values reported are uncorrected for recovery. For cell suspension, after thawing 0.5 ml of phosphate buffer was added, mixed well and 0.1 ml taken for radioimmunoassay (RIA).

## Results

Administration of A/S to human FSH or LH for a period of 8 days showed differential effects on estrous cycle. With LH antiserum treatment, the animals showed an estrous-diestrous smear, the FSH-antisera treated animals showed normal smear pattern for the first cycle and then showed a continuous atypical estrous; there was no significant decrease in ovarian and uterine weights (table 1). However, it can be

**Table 1.** Effect of antiserum to human follitropin and lutropin on ovarian and uterine wt and serum progesterone and estradiol -17 $\beta$

	Wt. of ovaries (mg)	Wt. of uterus (mg)	Serum	
			Progesterone ng/ml	Estradiol pg/ml
A NRS*	86.7±2.1	442.6±4.8	12.5 ±2.1	33.2±2.5
B FSH A/S treated	80.6±4.5	448.0±6.2	6.2 ±1.0	21.1±1.03
C LH A/S treated	77.6±1.2	303.0±11.2	1.96±0.5	10.8±1.50

All values are Mean ±S.E. of 5 observations.

Injections were given by subcutaneous route for a period of 8 days.

### P' Values

Ovaries	A-B	> 2.0
	A-C	< 0.025
Uterus	A-B	- N.S.
	A-C	< 0.005
Progesterone	A-B	< 0.025
	A-C	< 0.001
Estradiol	A-B	< 0.005
	A-C	< 0.001

\* Normal rabbit serum.

seen that neutralisation of LH by its antiserum was effective in decreasing the ovarian weights. The effects of depriving the endogenous gonadotropins were much more pronounced in the serum levels of progesterone and estradiol. Administration of FSH antiserum resulted in 50% and 37% decrease in the progesterone and estradiol -17 $\beta$  levels, respectively. Neutralisation of LH caused a drastic decrease in the serum progesterone level and nearly 70% decrease in the estradiol level.

The effects of *in vivo* neutralisation of FSH and LH on *in vitro* steroidogenic response of ovarian cells to exogenously added FSH and LH are presented in table 2. It can be seen that in the control group, FSH caused a significant stimulation of

**Table 2.** Effect of *in vivo* antiserum treatment of follitropin and lutropin on the response of rat ovarian cells to exogenous follitropin and lutropin.

Hormone	Progesterone pg/10 <sup>5</sup> cells			Estradiol -17 $\beta$ pg/10 <sup>6</sup> cells		
	NRS*	FSH A/S	LH A/S	LH A/S	NRS*	FSH A/S
	NRS*	FSH A/S	LH A/S	NRS*	FSH A/S	LH A/S
None	44.0 ± 1.0	27.7 ± 3.1	16.1 ± 0.9	7.1 ± 2.3	9.0 ± 4.0	0.25 ± 0.1
FSH	56.0 ± 0.8	35.9 ± 0.2	23.2 ± 1.4	14.0 ± 1.0	18.2 ± 0.4	0.5 ± 0.1
LH	327.5 ± 13.5	283.8 ± 9.0	126.4 ± 2.4	27.5 ± 14	17.9 ± 14	0.9 ± 0.5

Amount of FSH or LH used, 1 ng

All values are mean ± S.E. of triplicate determinations

\* NRS Norma rabbit serum

Progesterone and estradiol -17 $\beta$  determined by RIA (see text for details).

progesterone production. However, the stimulation was much more with the addition of LH. Neutralisation of FSH resulted in a significant decrease in basal production of progesterone as well as the response to both FSH and LH. As can be expected, the response to FSH in FSH A/S treated group is significantly decreased. There is a decrease in response to LH in the FSH A/S treated group. However, it is of interest that, in the LH A/S treated group, not only the basal production of progesterone is reduced but the response to both FSH and LH is significantly reduced.

The general picture for estradiol is similar to that of progesterone as far as the groups treated with normal rabbit serum or FSH A/S are concerned. However, neutralisation of LH resulted in a very drastic decrease in the basal production and in the response of both FSH and LH. The decrease in estradiol production is much more than in the case of progesterone.

## Discussion

The involvement of FSH in induction of ovulation and stimulation of steroidogenesis has been the subject of intense investigation (Nuti *et al.*, 1974; Jagannadha Rao *et al.*, 1974; Schwartz *et al.*, 1975). Thus far, all the reports concerned with the action of FSH on ovulation and steroidogenesis express some concern as to the specificity of the effects observed. However, recent studies have indicated that FSH itself has some intrinsic steroidogenesis activity in the complete absence of LH. The results of the present study using highly purified human FSH and LH and their antisera also supported such a conclusion. However, this activity is greatly reduced with prior treatment of the animal with LH A/S indicating that LH has a very significant role in the regulation of steroidogenesis. It is also possible that the decreased response of cells to FSH *in vitro* after neutralisation of LH *in vivo* may also be due to decreased FSH receptor content, which may in turn be due to decreased production of estrogen which facilitates induction of FSH receptor (Ireland *et al.*, 1978). It can be seen that quantitatively the cells produce much more progesterone than estradiol and deprival

of LH resulted in a very significant decrease in estradiol production in response to either FSH or LH. In view of the fact that estrodiol is way down in the steroidogenic pathway, this observation suggests that LH exerts its action at an earlier step and thus controls estradiol production by regulating the supply of precursor. Studies of Sheela Rani and Moudgal (1978) have shown that the block of estrogen synthesis in the cycling hamsters by LH A/S treatment is at the level of androgen synthesis. They observed that the inhibitory effect could be reversed by supplementing with testosterone under *in vitro* conditions. However, the present results show that in the FSH A/S treated group, there is no significant decrease in the estrogen production in response to FSH. This appears to contradict the observation of Sheela Rani and Moudgal (1978) who demonstrated that FSH is necessary for aromatisation of testosterone. It should be pointed out that in the present study no attempt has been made to isolate the granulosa cells or luteal cell and to sacrifice all the animals on the same day of the cycle. In view of this, the cell population includes luteal cells which could have contributed to the increased estrogen which also partly explains the lack of decrease in the weight of uterus in the FSH A/S treated group (B-table 1). Both progesterone and estrogen play an important role in the physiology of reproduction in the female and by regulating the precursor supply LH plays an important role in steroidogenesis. This partly explains the reasons for the significant and drastic effects observed following LH deprival compared to FSH deprival.

### Acknowledgements

This work was supported in part by USPHS grant AM-6097. We thank J. D. Nelson for technical assistance.

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## Modulation of testicular lutropin receptors in the developing male rat

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MS received 3 January 1980

**Abstract.** In the developing male rat around 40 days of age, the testis appears to contain the maximum amount of lutropin receptors per unit weight. During this period, circulating levels of testosterone markedly increase without the concomitant major surges in lutropin levels. The increased sensitivity and responsiveness of tests to basal levels of circulating lutropin during this period is accompanied by enhanced serum prolactin levels suggesting that this hormone may be involved in this process. The finding that prolactin treatment of pubertal rats for 3 days induced the formation of more testicular lutropin receptors supports the above premise. However, short-term immunoneutralisation of endogenous prolactin did not significantly alter the specific binding of [<sup>125</sup>I]-labelled lutropin to testicular membranes. Interestingly, during development, a close correlation exists between receptor occupancy and capacity of the tissue to bind labelled lutropin. The apparent dissociation between serum lutropin levels, on the one hand and tissue occupancy and free receptor contents on the other, suggests that factors other than lutropin (presumably prolactin) are involved in the modulation of the sensitivity and the responsiveness of the testis to lutropin during early development.

**Keywords.** Radioreceptor assay; radioimmunoassay; tissue-bound hormone; prolactin; puberty; lutropin receptor; ontogeny.

### Introduction

Recent investigations in the modulation of the testicular lutropin (LH) receptors in the rat have demonstrated that these receptors undergo marked fluctuations under several experimental conditions (Dufau *et al.*, 1978). Thus, hypophysectomy, chronic administration of large doses of LH, human chorionic gonadotropin (hCG) or dexamethasone leads to 'down regulation of LH receptor' phenomenon accompanied by desensitisation of the testis (Thanki and Steinberger, 1976; Frowein and Engel, 1975; Sharpe, 1976; Hsueh *et al.*, 1976; Chen and Payne, 1977; Purvis *et al.*, 1977; Saez *et al.*, 1977; Raff, 1976). On the other hand, treatment with follicle stimulating hormone (FSH) has been shown to enhance significantly the LH receptors in the rat testis (Chen *et al.*, 1976; Ketelslegers *et al.*, 1978). Furthermore, there is now abundant evidence to show that prolactin (PRL) could augment testicular responsiveness to LH in terms of the steroid hormone, testosterone production implying a permissive role for PRL at the testis (Odell and Swerdloff, 1976; Bartke *et al.*, 1978).

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Abbreviations used: Lutropin (LH), human chorionic gonadotropin (hCG), Follicle stimulating hormone (FSH), prolactin (PRL), rat PRL (rPRL) Ovine PRL (oPRL); antiserum (a/s).

In the present study, the ontogeny of LH receptors in the normal developing male rat has been examined to explore the possible existence of a temporal relationship among serum LH, PRL, testicular LH-receptor and receptor occupancy. In addition, the effects of PRL administration and its depression by specific immunoneutralisation on LH receptors in short-term experiments are described. A preliminary account of the work has been reported earlier (Prasad, 1978).

## Materials and methods

### Hormones and chemicals

Human lutropin (hLH), hCG, ovine PRL (oPRL), rat PRL (rPRL) and radioimmunoassay (RIA) kit for rat LH used in the present study were obtained through the courtesy of the Hormone Distribution Officer, NIAMDD, NIH, Bethesda Maryland, USA. Sephadex gels were obtained from Pharmacia, Uppsala, Sweden. Carrier-free [ $\text{Na}^{125}\text{I}$ ] and [ $^3\text{H}$ ]-testosterone (sp. act 90-105 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks, UK. Testosterone-antisera used in the present study was a kind gift of Prof. N. R. Moudgal, Department of Biochemistry, Indian Institute of Science, Bangalore. All other reagents and chemicals were of reagent grade.

### Animals

The male rats of Wistar strain were housed in cages and fed on pelleted diet (Hindustan Lever Products, Bombay), and water *ad libitum*. They were exposed to a 14 h light 10 h darkness schedule.

### Antiserum to rat PRL

Specific antiserum (a/s) to rat PRL was raised in rabbits. The hormone (200  $\mu\text{g}/0.5\text{ ml}$ ) emulsified with an equal volume of Freund's complete adjuvant (Difco) was injected subcutaneously at weekly intervals for 4 weeks into the animal at multiple sites. The rabbits were bled through the ear vein 7 days after the last injection. The presence of antibodies in the serum was tested by Ouchterlony double diffusion analysis (Ouchterlony, 1967). The antiserum gave a single precipitin line with rPRL and did not cross-react with ovine growth hormone or rLH and failed to bind significant amounts of these hormones (iodinated with  $^{125}\text{I}$ ) as measured by the procedure of Moudgal *et al.* (1979). The titre of the antiserum was high since at equivalence point, 1.0 ml of it could neutralise 400  $\mu\text{g}$  of rPRL.

### Radioimmunoassay of rPRL

The serum PRL concentrations of rats of different ages were determined by the modified radioimmunoassay procedure as described by Moudgal *et al.* (1979). Briefly, the serum samples were first incubated at 37°C for 10-12 h with rPRL antiserum at an appropriate predetermined dilution which would bind 20-40% of [ $^{125}\text{I}$ ]-rPRL. At the end of this period, a known amount of the iodinated hormone (50,000 cpm, 1.0 ng) was added and the incubation continued for another 10-12 h at 37°C. The free and bound labelled hormones were separated by the addition of 1 ml of goat anti-serum to rabbit IgG (double antibody) along with 0.1 ml of 1:50 diluted normal rabbit serum added as carrier. The precipitate formed after a further incubation for 10-12 h at 37°C was collected by centrifugation and counted for radioactivity in a Packard Autogamma Spectrometer (model 2002). The serum samples were assayed

in duplicates at two different levels, and PRL levels were expressed as ng/ml in terms of NIAMDD-rat-PRL-RPL standard. The coefficient of variation for and between assays were 3% and 10% respectively.

#### *Radioimmunoassay of testosterone*

The method was based essentially on the procedure described earlier for other steroids (Niswender *et al.*, 1975). Briefly, the serum samples were extracted twice with 5 volumes of diethyl ether and the ether layer aspirated into separate tubes after freezing the lower aqueous (protein) phase in liquid nitrogen. The pooled ether extracts were evaporated in a water bath at 45°C and reconstituted with 1.0 ml of RIA buffer (0.01 M sodium phosphate buffer, pH 7.5 containing 0.9% NaCl+1% gelatin). Routinely, RIA for testosterone was carried out in triplicate. In 10×75 cm glass tubes were included, 100 µl of either the steroid fractions extracted from serum samples, or testosterone standards. Then, 100 µl of [<sup>3</sup>H]-testosterone (10,000 cpm) and 100 µl of 1:4,000 diluted testosterone-a/s were added. The total volume was made up to 500 µl with the RIA buffer and the tubes incubated at 4°C for 6 h. The nonspecific binding of [<sup>3</sup>H]-testosterone was determined by omitting the a/s, whereas the total binding to the a/s was computed by omitting the non-radioactive testosterone standard.

At the end of the incubation, free and bound testosterone were separated by adding 500 µl of dextran-coated charcoal (0.05% dextran-70 and 0.5% acid-washed charcoal in RIA buffer without gelatin). The contents of the tubes were quickly mixed and incubated at 4°C for exactly 10 min. They were then centrifuged at 5,000 × g for 10 min at 4°C. The radio-activity in supernatants was measured using toluene-triton x-100 scintillation cocktail in a Beckman LS-100 liquid scintillation spectrometer.

The nonspecifically bound radioactivity was always deducted from the total bound radio-activity and the standard curve constructed by plotting pg of testosterone against the % specific binding. The sensitivity of the RIA was in the range of 5 to 10 pg.

#### *Preparation of [<sup>125</sup>I]-human lutropin for receptor assay*

The chloramine-T method of Hunter and Greenwood (1962) was not suitable for preparing the labelled hLH for receptor assays. However, a slight modification of this method to minimise the damage, reproducibly yielded [<sup>125</sup>I]-hLH suitable for hormone-receptor binding studies. Briefly, the iodination reaction was performed in a 10×75 mm glass tube kept in crushed ice. To the tube containing hLH (20 µg) approximately 500 µCi of Na<sup>125</sup>I was added. The iodination reaction was initiated by the addition of 50 µl of 1 mg/ml chloramine-T and allowed to continue for 30 sec with occasional shaking. The reaction was then stopped by the addition of 50 µl of 5 mg/ml sodium metabisulphite solution. The entire reaction mixture was applied to a Sephadex G-75 column (15 ml bed volume), pre-equilibrated in 0.05 M Tris-HCl (pH 7.6) containing 0.15 M NaCl and 0.5% ovalbumin. The column was eluted with the same buffer and the eluent collected in 0.5 ml fractions. The peak fraction containing labelled hormone was used directly for the receptor assays and had a specific activity of 75,000 cpm/ng protein.

### *Preparation of receptor-rich membranes*

The crude membrane used for testicular receptor assays was prepared as follows: rats of different ages (3-4 animals/group) were killed by decapitation, testis excised and weighed. The decapsulated tissue was minced, and homogenised in a ground-glass homogeniser by hand at 0-4°C using 10 vol of (w/v) 0.02 M Tris-HCl (pH 7.6) buffer containing 0.15 M NaCl. Receptor-rich pellets were obtained by centrifugation at 20,000 g for 20 min. The membranes were washed three times with the initial buffer and finally resuspended in 0.02 M Tris-HCl (pH 7.6) buffer containing 0.15 M NaCl and 10 mM MgCl<sub>2</sub>, such that the membrane concentration corresponded to 100 mg wet weight of testicular tissue per ml of the buffer. These preparations were immediately used for the assay (Reichert *et al.*, 1973).

### *Incubation procedure for the assessment of [<sup>125</sup>I]-human lutropin binding*

Incubations were performed at room temperature in 10 mm×50 mm glass tubes in a total volume of 0.5 ml. Both the labelled hormone and the membrane preparations were diluted using 0.02 M Tris-HCl (pH 7.6) buffer containing 10 mM MgCl<sub>2</sub>, 0.15 M NaCl and 0.1% ovalbumin. The binding reaction was initiated by adding an aliquot of approximately 1-1.5 ng [<sup>125</sup>I]-hLH ( $7.5 \times 10^4 - 1 \times 10^5$  cpm) to the membrane preparations. Incubations were performed for 12 to 16 h in all the experiments. Binding assays were conducted at two different concentrations of the membranes (equivalent to 10 and 20 mg tissue/tube) in triplicate. Reactions were terminated by dilution with the cold (5°C) buffer (1.0 ml) followed by centrifugation at 20,000 g for 30 min. The supernatants were discarded and the radioactivity in the pellets was measured in a Packard Autogamma Spectrometer (Model 2002).

Total binding refers to the radioactivity bound to the pellet in the absence of added unlabelled hormone. Non-specific binding is represented by the radioactivity bound in the presence of 1000-fold excess (1 µg) of unlabelled hormone. Specific binding was obtained by subtracting the nonspecific from the total binding, and was expressed as per cent of the total radioactive hormone added to the incubation tubes. The nonspecific binding was 6 to 8%.

In separate experiments, it could be shown that the specific binding of [<sup>125</sup>I]-labelled LH to the testicular membranes was not influenced by 1 µg each of oPRL and ovine growth hormone (oGH) further showing the specificity of the binding of labelled hormone. The Scatchard analysis of the binding data with 40 day old rat testis preparation showed that the binding occurred with  $K_a = 0.25 \times 10^{10} \text{ M}^{-1}$  and the plots were linear.

### *Measurement of tissue-bound and serum levels of lutropin*

Testis-bound LH was measured by radioimmunoassay carried out at the elevated temperature of 37°C (Muralidhar and Moudgal, 1976; Sheela Rani and Moudgal, 1978). Briefly, the decapsulated testes were homogenised in 5 vol (w/v) of 0.05 M sodium phosphate buffer (pH 7.4), containing 0.5 M EDTA and 0.15 M NaCl at 0-4°C. The homogenate was filtered and suitable aliquots were directly used for LH assay. All samples were assayed at two different levels and the amounts of labelled LH nonspecifically bound in the absence of any added a s was determined.

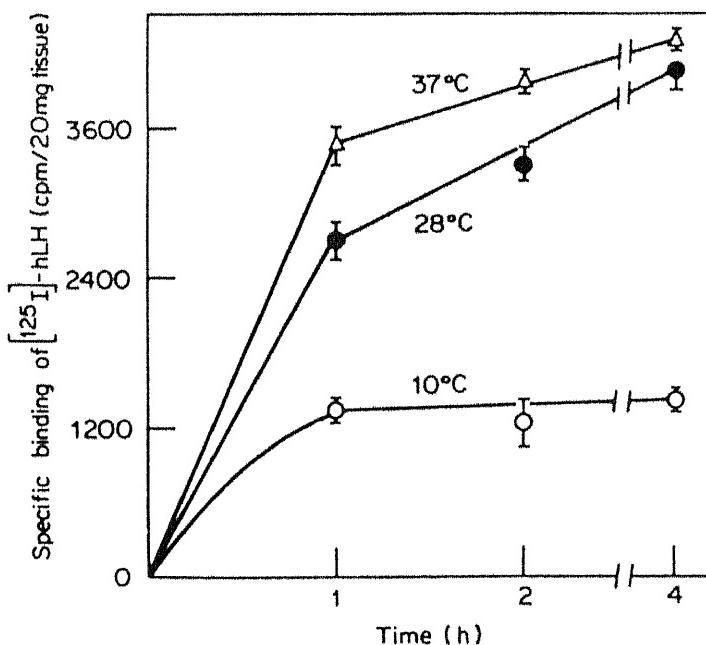
LH in the tissue homogenates and serum samples was measured by the modified RIA procedure using NIAMDD rat LH-RIA kit. Incubations at all stages of the RIA were carried out at 37°C. Briefly, the procedure consisted of incubating the tissue

or serum samples with the rat LH a/s for a period of 10-12 h at 37°C. This was followed by the addition of [<sup>125</sup>I]-labelled rat LH and a further incubation at 37°C for 10-12 h; then goat antibody to rabbit IgG (double antibody) was added to precipitate the bound label and the incubation continued for an additional period of 10-12 h. At the end of this incubation, the tubes were centrifuged at 3,000 g for 30 min and the precipitate obtained was used for bound radioactivity measurement. It was independently determined that the intraassay variation was minimal (8%) and non-target tissue homogenates did not interfere in the assay (Sheela Rani and Moudgal, 1978). The validity and reproducibility of the above mentioned modified RIA method to quantitate the tissue bound hormones have been amply demonstrated earlier by others in this laboratory (Muralidhar and Moudgal, 1976; Sheela Rani and Moudgal, 1978). Recovery experiments have revealed that there was no loss of LH during incubation at 37°C.

## Results

### *Influence of time and temperature on the specific binding of [<sup>125</sup>I]-hLH to testicular membranes*

As shown in figure 1 the binding of labelled hLH to testicular membranes was dependent on time and temperature of incubation. At 37°C, equilibrium was attained after 4 h of incubation. Although the rate of specific binding was initially lower at

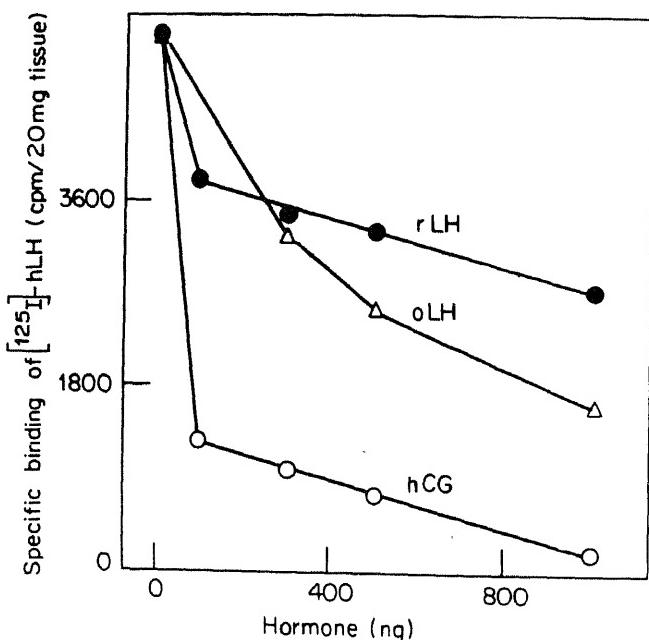


**Figure 1.** Effect of time and temperature on the specific binding of [<sup>125</sup>I]-human LH to the testicular membrane preparation. Crude testicular membranes from five animals were prepared and incubated at 0°C, 28°C and 37°C for different periods with [<sup>125</sup>I]-human LH ( $50-70 \times 10^3$  cpm) in triplicate. Nonspecific binding was determined for each set by including 1  $\mu$ g of hLH. Data presented are Mean  $\pm$  S.D. ( $n=5$ ). The experiments were repeated three times with similar results.

28°C, the binding increased with time and ultimately at 4 h approached the values obtained at 37°C. On the other hand, specific binding continued to remain low (~ 50%) at 0°C even after 4 h of incubation. Further studies have shown that incubation at room temperature (28°C) for a period of 12-16 h could give maximum specific binding and therefore most of the studies reported hereafter were carried out by incubating membrane preparations with [<sup>125</sup>I]-hormone for 12-16 at 28°C.

#### *Displacement of labelled lutropin by unlabelled hormones*

To demonstrate the specificity of hormonal binding, the testicular membrane preparations were incubated with fixed amount of [<sup>125</sup>I]-hLH with varying amounts of unlabelled hormones. Data presented in figure 2 clearly show that all the three hormones (hCG, ovine LH, and rat LH) could compete for the binding sites and that among these, hCG was the most effective. The unequivocal ability of these hormones to compete effectively for the binding sites testify the physiological relevance of this receptor interaction. Further, as low as 100 ng hCG was sufficient to cause 50% reduction in the specific binding, while a 10-fold excess (1000 ng) of LH was needed for a comparable effect.

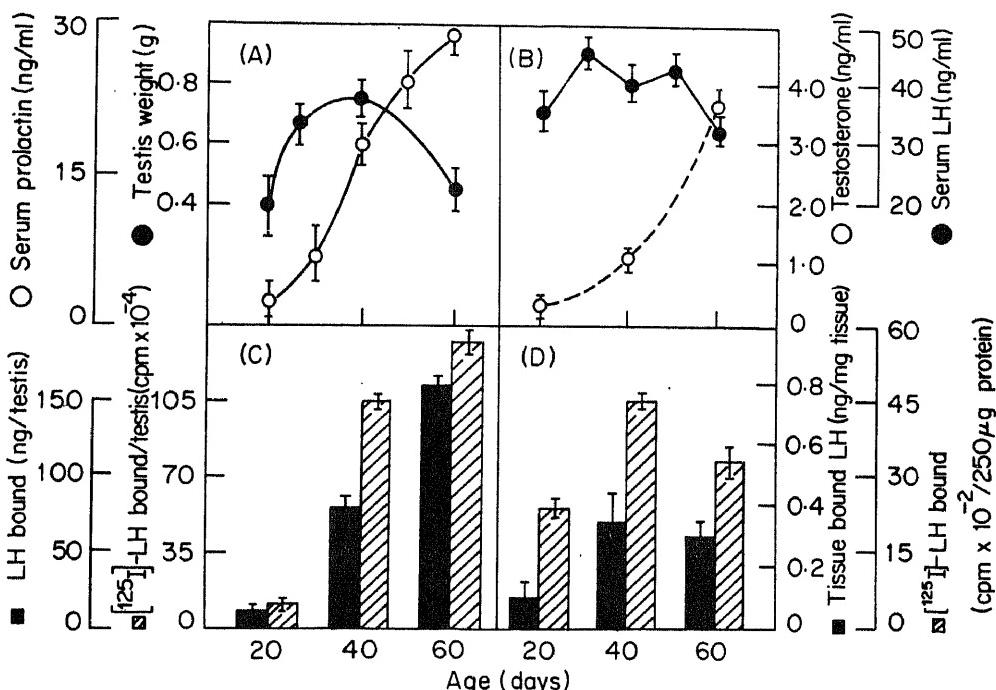


**Figure 2.** Displacement of [<sup>125</sup>I]-human LH from testicular receptors by unlabelled hormones. The optimal conditions for hormone binding were described earlier. Binding assay was carried out in duplicate in the presence of known amounts of unlabelled hormones (hCG, ovine LH or rat LH) as competing species. Labelled hormone bound to membrane in the presence of 5 µg of these hormones was taken as the corresponding nonspecific binding. For further details see text. The experiments were repeated three times employing six animals each time with essentially similar results.

*Ontogeny of testicular lutropin receptors—relation to circulatory lutropin, testosterone and occupancy*

In order to investigate the developmental changes in the LH receptor content of the testicular tissue, receptor content in testicular membrane preparations from groups of rats of different age were determined. The circulatory and tissue-bound LH levels and serum testosterone concentrations as well as the fresh weights of testis were determined to examine the possible relationship among these parameters. It may be seen (figure 3) that testicular weight increased steeply (nearly 10-fold) between 20 and 40 days of age and continued to increase thereafter, though at a much reduced rate. During this time, there was no significant change in serum LH levels while circulatory testosterone levels (figure 3) showed a mere two-fold increase by day 40 which was followed by a more dramatic increase thereafter.

To gain an insight into this increased ability of the testis (despite exposure to basal levels of circulatory LH) to elaborate enhanced steroid hormone *in vivo* after 40 days of age, it was important to examine the ability of the tissue to bind LH. When the total tissue contents of LH and free LH receptors were measured, the picture



**Figure 3.** Ontogeny of testicular LH receptors—relation to circulatory LH, T and occupancy.  
 A. Fresh weights of testis and serum PRL levels in 20, 40 and 60-day old rats. Data presented are the Mean  $\pm$  S.D. of 3 animals/group. B. Serum T and LH levels measured by the specific radioimmunoassays. Data experiments were repeated 4 times with similar results. C.  $[^{125}\text{I}]$ -hLH total binding capacity/testis from 20, 40 and 60-day old rats. LH bound to testis of 20, 40 and 60-day old rats, measured by the modified RIA. The data represent Mean  $\pm$  S.D. of 5 animals/group. D.  $[^{125}\text{I}]$ -LH binding capacity/unit membrane protein. Amount of LH bound *in vivo* (occupancy) to the testicular membrane protein ( $250\ \mu\text{g}$ ). The data represent Mean  $\pm$  S.D. of 5 animals/groups. For further details see text.

that emerged is summarised in figure 3C. It may be seen that with increase in age an almost parallel enhancement in tissue-bound LH as well as total free receptor contents were clearly evident. The increased tissue-bound LH (occupancy) accompanied by corresponding increase in the free receptor content are in excellent agreement with observed increased production of the androgen. Further, a closer examination of figure 3C shows that at 40 days of age, there appears to be a relatively greater number of receptors available for LH binding compared to day 20 or 60. This probably explains the greater capacity of the testis to respond to LH acquired in the course of development of the animal (around day 40). Supporting evidence stems from the results given in figure 3D, pertaining to the capacity of unit testicular membrane protein to interact specifically with [<sup>125</sup>I]-hLH. It is clear that there was a marked increase in the ability of the membrane preparations to bind LH around 40 days of age to be followed by a marginal decrease thereafter. Interestingly, parallel changes were observed in the case of tissue-bound LH also.

#### *Modulation of testicular lutropin receptors by prolactin status of the animal*

An examination of serum PRL levels during development revealed the existence of peak levels of this hormone around 40-50 days of age (figure 3B) suggesting that the elevated levels of this hormone (PRL) might have altered the ability of the testis to respond to LH at this stage. In order to establish the possible involvement of PRL in the enhancement of free LH receptors in the testis, 60 day-old rats were treated for 3 days with oPRL (1 mg/100 gm body weight) or a potent a/s to rPRL. It is clear from table 1 that treatment with oPRL could in fact significantly increase the LH-binding

**Table 1.** Change in LH receptor contents in the testis following treatment with ovine PRL and rat PRL a/s *in vivo* in the male rat.

Treatment	[ <sup>125</sup> I]-hLH bound (cpm)/20 mg tissue (Mean ± S.D.)
None (control)	7239±289
Ovine PRL	11620±321*
PRL a/s	7892±140

Sixty-day old male rats (4 animals/group) were treated for 3 days with PRL (1 mg/day/100 gm body weight) or rat PRL a/s (0.2 ml/day/rat). Specific binding of [<sup>125</sup>I]-hLH to testicular membranes prepared from each animal was determined as described in the text. Control animals receiving 0.2 ml/animal of normal rabbit serum had their testicular LH receptors equal to untreated controls. The experiment was repeated three times with similar results.

\* Significant compared with control ( $P < 0.01$ ).

capacity of the testis. However, the deprivation of PRL by a/s treatment for 3 days had no marked effect on testicular LH-binding capacity.

The amount (0.2 ml/day) of the potent rPRL a/s administered to each animal would have neutralised 80  $\mu$ g of the hormone *in vitro*, and is therefore considered sufficient to neutralise a large proportion of the endogenous hormone whose levels were around 20 ng/ml, the highest during the development (figure 3).

## Discussion

The developmental changes in testicular LH receptors, circulatory LH and PRL were determined in the male rat to examine the possible mode of interaction between these two hormones at the receptor levels on the Leydig cells culminating in a dramatic increase in serum testosterone levels after 40 days of age. This incidentally also provided an opportunity to validate several recent claims (Bartke *et al.*, 1978) that PRL directly influences testicular function. Following systematic characterisation of specific LH receptors on testicular membranes, the modulation of their contents during developments in relation to circulatory gonadotropin (LH) levels was examined particularly in view of the recent findings (Lesniak and Roth, 1976) that the primary regulators of the availability of receptors in the respective target tissues are the corresponding hormones themselves. Our data (figure 3) on LH receptor and serum testosterone levels in the developing male rat are of interest in this connection as they show the absence of any correlation between serum LH levels (nearly constant) and the corresponding contents of either the total tissue-bound LH or the free hormonal receptors. The content of both tissue-bound and free LH receptors markedly increased with the age of the animal accompanied by an increase in testicular weight which may be reflection of increased number of Leydig cells during development (Ketelslegers *et al.*, 1978). Low levels of tissue-bound LH as well as the free LH receptors (total as well as per unit weight) thus clearly explain the relative refractoriness of the immature (20 day) rat testis to LH. More intriguing are the data concerning the lack of parallelism between the increase in serum testosterone contents and corresponding dramatic enhancement in testicular weights (>10-fold; figure 3) during 20-40 days of age (unlike during 40-60 days of age). The most likely explanation for this lag in the steroid hormone output by the testis during the earlier days is provided by the results depicted in figure 3C, which show nearly doubling of the "specific activity" of both the [ $^{125}$ I]-LH binding (i.e. per unit tissue weight) and the tissue-bound LH (i.e. per unit membrane protein) around day 40, followed by a slight decrease around day 60. In other words, the marked increase in the capacity of the unit weight of the testis to interact with available LH appears to enable the tissue to harbour enhanced amount of LH from circulation (figure 3C). This greater ability acquired may be one of the factors contributing to the secretion of relatively larger amounts of testosterone after 40 days. This enhanced capacity is, in fact, disproportionate to the testicular weight increments (hence presumably to increase in number of Leydig cells) thereafter. Supporting evidence for this premise stems from the data of figure 3C which show that around day 40, relatively greater amount of total free LH receptors are available than are occupied (by tissue bound LH) *vis-a-vis* days 20 or 60. But around day 60, a relatively greater proportion of measurable receptors are occupied by the hormone, thus explaining the pronounced increase in circulatory testosterone. However, at this time, the capacity of the unit membrane protein to interact with [ $^{125}$ I]-LH seems to be slightly reduced. The pattern *viz.*, relatively low levels of total tissue bound LH around day 20, followed by marked increase around day 40, registering further increase thereafter appears to be in concert with the corresponding levels of circulatory testosterone.

One of the likely candidates responsible for initiating this marked change in testicular capacity to bind LH and hence to elaborate increased amount of testosterone appears to be PRL. Presumptive evidence for this postulate is provided by the elevated levels of serum PRL around 40-50 days of age (figure 3). The demonstration that exogenous PRL significantly enhanced both the specific (per unit membrane protein) and total testis LH receptor content is in agreement with the finding that enhanced PRL levels in circulation coincides with augmented testosterone production *in vivo* in 60 days old rat. Pertinent at this juncture are the data of Zipf *et al.*, (1978) showing that in hypophysectomised rats, chronic PRL treatment alone prevented the loss of LH receptor whereas PRL in combination with low levels of LH enhanced the testicular LH receptors. It is realised that enhanced LH receptor content and occupancy alone is not sufficient to stimulate testosterone production by Leydig cells and that biosynthetic processes should also be accelerated. It is relevant to recall that increased responsiveness to exogenous LH during sexual maturation (Odell *et al.*, 1974) coincided with increased LH receptors (Ketelslegers *et al.*, 1978). Also of significance in this context are the findings of others (Hafiez *et al.*, 1971; Musto *et al.*, 1972) that PRL increases precursor availability and stimulates some of the enzymes ( $3\beta$  and  $17\alpha$ -hydroxy steroid dehydrogenases) involved in androgen production.

It is noteworthy that short-time PRL deprivation by immunological neutralisation did not significantly alter LH receptor content. Inadequate neutralisation does not appear to be a likely explanation for this negative result since the dosage of the potent PRL a/s would have been adequate to neutralise around  $80 \mu\text{g}$  of circulatory rPRL/day. Thus it would appear that PRL is dispensable at least for short duration in terms of the maintenance of LH receptor content and this may be related to the turnover rate of the receptors. Also noteworthy is the demonstration of Hauger *et al.* (1977) that selective reduction of plasma gonadotropins for 3-6 days by immunoneutralisation with anti-LHRH serum had no effect on testicular receptor. But Aragona *et al.* (1977), employing 2-bromoergocryptine (CB-154) to inhibit PRL release in immature rats have shown significant reduction in testicular LH receptors. The reasons for this apparent discrepancy between our data and those of Aragona *et al.* (1977) are not clear at present and may be related to such factors as the age/strain of the animals and/or the duration and modes (drug vs antibody) of PRL deprivation employed.

However, even though the testis of PRL-treated rats displayed higher LH receptor contents, their responsiveness *in vitro* to high level of LH ( $10 \mu\text{g}/\text{ml}$ ) was not significantly different from the control tissue in terms of steroidogenic capacity ( $0.4 \pm 0.025 \text{ ng}/\text{h}/\text{ml}$  medium/g wet tissue, control vs  $0.45 \pm 0.03 \text{ ng}/\text{h}/\text{ml}$  medium/g wet tissue, PRL-treated). Since only a small portion (1%) of total available LH receptors need be occupied for maximal steroidogenic response (Dufau *et al.*, 1978) this could have been easily accomplished under *in vitro* conditions employing supraphysiological doses of LH. While a strict correlation between receptor contents and testicular responsiveness to LH has not been demonstrated with an intact tissue, it is conceivable that under *in vivo* conditions, PRL-induced elevated LH receptor content may be of physiological relevance as a possible mechanism enabling the testis to harbour more of LH and hence respond progressively to a greater extent (Dufau and Catt 1978) in the absence of any major surges in the circulatory LH levels. The demonstration of specific PRL receptors on Leydig cells (Aragoná *et al.*, 1977) on which LH acts to stimulate testosterone production is of relevance in this context.

Since there is no evidence for an effect of PRL on Leydig cell numbers, it appears that PRL increases the number of LH receptors per Leydig cell.

The data presented here, however, do not exclude other physiological factors exerting coordinated influence in enhancing testicular responsiveness to available LH. For example, Odell and Swerdlow (1976) have suggested that FSH has a significant role to play in this regard especially during sexual maturation. High circulatory levels of FSH (Nandini *et al.*, 1976; Ketelslegers *et al.*, 1978) during days 21 or 32 in the male rat appear to be an essential factor to be reckoned with in this context. More recent demonstration that chronic FSH administration to 15-day old immature rats significantly enhanced total testicular LH receptors which was not strictly proportional to increase in testicular weight suggests that the FSH is involved in the induction of LH receptors (Chen *et al.*, 1977; Marah, 1976). But there was no temporal relationship between LH receptor content and *in vitro* testicular responsiveness to LH under these conditions, and hence the relevance of these observations to the data described herein is not clear at present. It is conceivable that during early developmental period of the male rat, multiple hormones interact coordinately at the testicular level to enhance the tissue capacity to secrete the androgen by increasing either the number of total Leydig cells, LH receptor content or the capacity of the tissue to bind and respond to LH.

### Acknowledgements

We are highly thankful to Mr. K. Rama Sharma for his help in carrying out the tissue-bound hormone assays. Our thanks are due to Prof. N. R. Moudgal for helpful discussions and facilities for radioimmuno and radioreceptor assays. One of us (MSKP) thanks the University Grants Commission, New Delhi, for the financial assistance.

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**Statement about ownership and other particulars about  
"Journal of Biosciences"**

1. Place of Publication	... Bangalore
2. Periodicity of Publication	... Quarterly
3. Printer's Name	... S. Ramaseshan Indian Academy of Sciences, Bangalore 560 080
4. and 5. Publisher and Editor	... S. Ramaseshan
5. Nationality	... Indian
6. Address	... Indian Academy of Sciences P.B. No. 8005, Bangalore 560 080
7. Name and Address of the Owner	... Indian Academy of Sciences BANGALORE 560 080

I, S. Ramaseshan, hereby declare that the particulars given above are true to the best of my knowledge.

Date: 1st March 1980

S. Ramaseshan  
*Signature of Publisher*

## **Adaptation to salinity by fish: Alterations in energy transducing status of muscle mitochondria**

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MS received 3 November 1979; revised 8 March 1980

**Abstract.** The functional status of fish muscle mitochondria during exposure of the organism to salinity stress was studied. No alterations were observed in the substrate oxidation capacity. However, the mitochondria appear to be in an uncoupled state during the first few days of exposure and recover thereafter. This could be correlated to high endogenous  $\text{Ca}^{2+}$  levels in the mitochondria in the early period. A  $\text{Na}^+ - \text{Ca}^{2+}$  antiport in these mitochondria is shown. Evidence is also presented to show that the mitochondria isolated during the early stages of stress are in a swollen state and are unable to contract on addition of ATP and  $\text{Mg}^{2+}$ . Continued exposure to the stress, however, reverses the situation.

**Keywords.** Fish; salinity adaptations; muscle mitochondria; respiratory status; calcium uptake swelling and contraction.

### **Introduction**

The classical picture of mitochondria as the ATP synthesizing unit is fast being replaced by one envisaging it as a multi-functional unit, which transduces the chemical energy of substrate oxidation to different functions, like ATP synthesis, ion uptake, conformational changes, proton pumps, active transport etc. Thus there is *prima facie* evidence for channelized transduction and the question arises as to whether this channelization is regulated by the physiological and environmental exigencies of the cell.

A survey of mitochondria isolated from muscle tissues of some arthropods (Honnappa *et al.*, 1975) and from the mantle tissues of molluscs (Hannah Sulochana *et al.*, 1977a) had shown a possible correlation between the  $\text{Ca}^{2+}$  uptake capacity and the calcium need of the organism. Carafoli and Lehninger (1971) have also compared the interaction of  $\text{Ca}^{2+}$  with mitochondria from different tissues and species in an attempt to understand the relevance of calcium uptake by mitochondria. Another comparative survey in this laboratory (Honnappa, unpublished results) of the mitochondrial properties from different organisms, gave preliminary evidence for channelization of energy transduced and this varied with the physiology of the organism. We have also reported earlier (Hannah Sulochana *et al.*, 1977b) that mitochondrial functions respond to environmental stress conditions imposed on

the organism. In this study, the fresh water fish *Tilapia mossambica* (now renamed *Sarotherodon mossambicus*) was exposed to iono-osmotic stress, by transferring them from fresh water to 25% sea water. At various stages of transfer, we have isolated the mitochondria from the muscle tissue and studied the energy transducing functions of the organelle. Since the stress imposed was iono-osmotic in nature, we have concentrated our attention on two major energy transducing functions of the organelle, namely calcium uptake swelling and contraction.

### Materials and methods

The conditions for collection of fish, acclimation to laboratory conditions (fresh water media) and direct exposure to stress conditions were as described earlier (Hannah Sulochana *et al.*, 1977b).

#### *Isolation of mitochondria*

The procedure used was the same as that reported earlier (Hannah Sulochana *et al.*, 1977b). The final mitochondrial pellet was washed once and suspended in 0.25 M sucrose. For swelling and contraction studies, 0.05 to 0.1 ml of the suspension (approximately 1 mg protein/ml) in 1 ml medium with an absorbancy of 0.4 at 520 nm were used.

#### *Analytical methods*

Protein was measured by Lowry's method using bovine serum albumin as the standard (Lowry *et al.*, 1951). In the case of sucrose density gradient analysis, Bradford's method (Bradford, 1976) was used for obtaining the protein profile.

Oxygen uptake was measured polarographically using a Clark type electrode attached to an Elico (India) X-Y recorder. The reaction medium and other conditions were the same as that reported earlier (Honnappa *et al.*, 1975). For the NaCl treatment, samples containing the same amount of mitochondrial protein used for the polarographic assay were taken. NaCl (20 mM) was added and the mixture was incubated for 3 min, followed by centrifugation at 10000 g in a Janetzki TH 12 centrifuge for 5 min. The pellets were washed twice with buffer and then used for oxygen uptake studies.

$Mg^{2+}$ -ATPase activity was estimated according to the method of Epstein and Whittam (1966). The reaction medium (1 ml) contained 2 mM  $MgCl_2$ , 2 mM ATP, 8 mM imidazole acetate (pH 7.4). Mitochondria were added to initiate the reaction. After 10 min, the reaction was stopped by the addition of 0.3 ml of ice cold 10% trichloroacetic acid. The samples were kept in ice for 10 min and then centrifuged for 2 min at 10000 g. Inorganic orthophosphate in the supernatant was estimated according to the method of Fiske and Subbarow (1925). For the density gradient analysis, a discontinuous sucrose gradient containing 20, 30, 40, 50, 60, 65, 75% sucrose (total volume 4.5 ml) was used. Mitochondria, 0.1 ml—0.2 ml. (about 560  $\mu$ g protein) was loaded. The centrifugation was carried out at 108000 g in a VAC 601 ultracentrifuge for 1 h. Fractions were collected and succinate dehydrogenase and protein estimations were made in each sample.

For the measurement of endogenous ion level, mitochondria were extracted with 0.5 N HCl by boiling in a water bath for 15 min. The extract was centrifuged at 10000 g for 5 min. and the ions  $K^+$ ,  $Na^+$  and  $C2^+$  were estimated in the supernatant by flame photometry.

The swelling experiments were performed according to Richardson and Tappel (1962) with slight modifications. Sucrose solutions were prepared in deionised, double distilled water. Mitochondria were added to the required sucrose media and swelling was measured by the decrease in absorbance at 520 nm in a Beckman DK-2 ratio recorder. Temperature was maintained at a constant value. The rate of swelling was calculated from the slope of the curve. Contraction of the swollen mitochondria was produced by the addition of a mixture of 5 mM ATP and 5 mM MgCl<sub>2</sub> after swelling the mitochondria in the sucrose medium.

## Results

### *Respiratory capacity*

Fishes were transferred from fresh water (tap water with negligible ionic concentration) to 25% sea water as mentioned in materials and methods. After different days of exposure, samples of fishes were taken out, muscle tissue excised out and mitochondria were isolated. The results obtained with muscle mitochondria are given in table 1. As can be seen, upto 14 days of exposure, no significant variations were observed

**Table 1.** Respiratory status of muscle mitochondria during long term exposure to 25% sea water stress.

Mitochondria†	Treatment	natoms oxygen uptake/ units min/mg. mitochondrial protein		ADP/O	R.C.	Mg <sup>2+</sup> dependent ATPase levels, (umol Pi/min/mg protein)
		Succinate	+ Dinitrophenol			
Fresh water		5.8	11.5	1.8	2.0	0.74
1 day		6.3	6.3	0	1.0	
2 days		6.4	6.4	0	1.0	0.33
7 days		5.7	5.7	0	1.0	0.33
9 days		4.2	5.4	0	1.0	0.46
14 days		6.8	11.4	1.7	2.0	0.50
24 days		6.4	11.5	1.8	2.0	0.70
Fresh water	Calcium loading	5.8	n.d.	0	1.6	n.d.
7 days	NaCl	5.7	8.4	1.6	2.0	n.d..
9 days	NaCl	6.3	8.4	1.5	2.0	n.d.

† — The period of exposure to 25% sea water

n.d — Not determined

R.C. — Respiratory control index

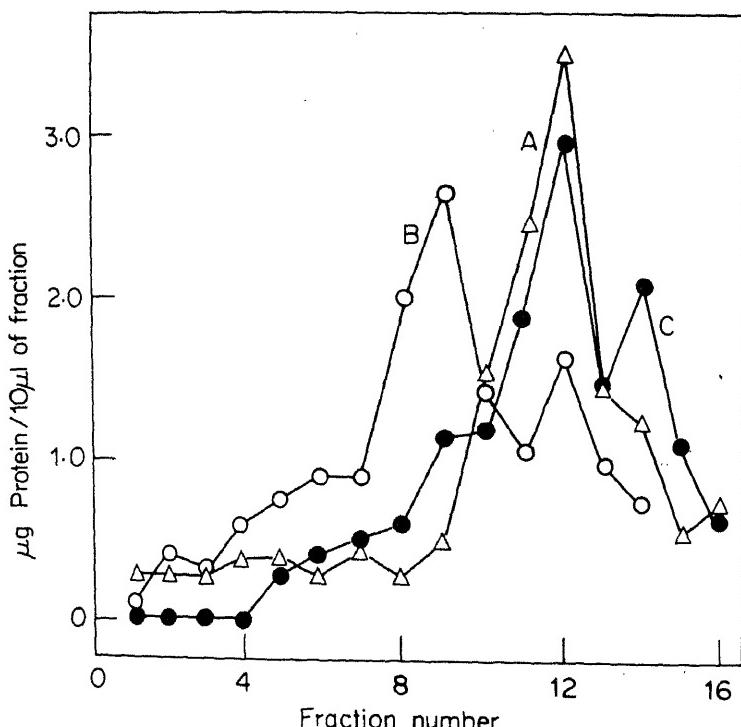
in the succinate oxidation rate (state II). However, a different picture was seen when the dinitrophenol stimulated respiration rate (state V), respiratory control index and ADP/O ratios were taken into account. Starting on the very first day of exposure, the parameters were not measurable upto about the ninth day. But on subsequent exposure, on the 14th day, stimulation by dinitrophenol, respiratory control index and ADP/O ratios were restored to normal values.

### *ATPase activity*

The  $Mg^{2+}$ -dependent ATPase activity during the course of stress is also given in table 1. It is obvious, that there is a drastic reduction (two-fold) in the activity soon after exposure to stress. The activity remains low till about the ninth day and thereafter recovers back to the control (fresh water) level rapidly by about the 24th day. These results fit well with the changes observed in ADP/O ratio and respiratory control values as mentioned above. The enzyme activities in post-mitochondrial supernatant fractions were also measured throughout and they remained at a steady low value of  $0.1 \mu\text{mol P}_i/\text{min/mg protein}$  throughout the period.

### *Density gradient analysis*

The various mitochondrial samples were also subjected to sucrose density gradient centrifugation for further characterisation. The density gradient profiles are shown in figure 1.



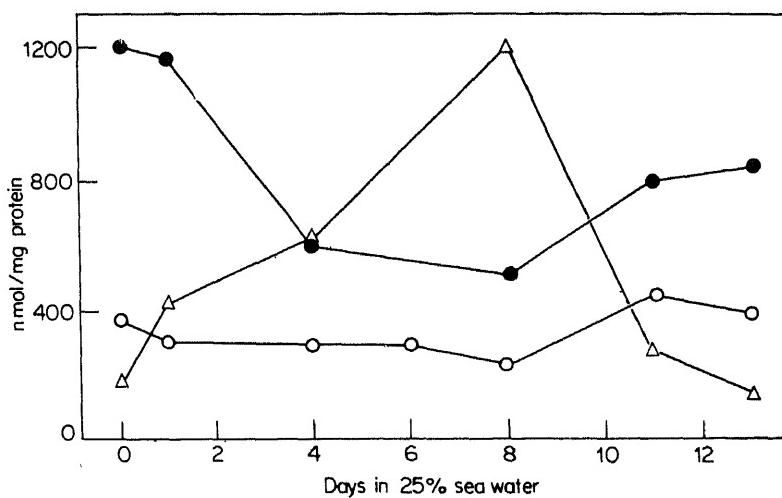
**Figure 1.** Density-gradient analysis of Tilapia muscle mitochondria. (A) in fresh water. (B) On 7th day in 25% sea water. (C) Adapted to 25% sea water (exposed for more than 25 days).

Mitochondria from fresh water fish tissues showed essentially a single peak with succinate dehydrogenase activity at a density of  $1.2175 \text{ g/cm}^3$ . There was a small shoulder with no enzyme activity. Thus the preparations used in the studies were fairly pure. Mitochondria from muscle tissues of fishes exposed to 25% sea water for 7 days, however, showed two peaks at densities of  $1.2296 \text{ g/cm}^3$  and  $1.2175 \text{ g/cm}^3$ , and both peaks had succinate dehydrogenase activity. When mitochondria were isolated 25 days after exposure the pattern was shifted. The heavier peak shifted to low density corresponding to that of fresh water samples and there was also a lighter peak (density  $1.2170 \text{ g/cm}^3$ ) containing succinate dehydrogenase activity.

One possible reason for the lighter density mitochondria becoming heavier, could be the loading of mitochondria with calcium (Lehninger, 1965). That this indeed is the case was shown by measuring of  $\text{Ca}^{2+}$  levels in the mitochondria (see below). A proportion of the mitochondria remained free of  $\text{Ca}^{2+}$  which is reflected by the second peak (density  $1.2175 \text{ g/cm}^3$ ). It was interesting to find that on longer exposure to stress, the mitochondrial density returned to the normal value with the concomitant decrease in calcium levels (see also figure 2). The appearance of a peak at lower density  $1.2170 \text{ g/cm}^3$  with succinate dehydrogenase activity could support the contention of new mitochondria being formed in this phase but this must await further experimentation.

#### *Endogenous levels of ions*

A study of the levels of different ions in these mitochondria was next carried out (figure 2). There were significant changes in the levels of  $\text{Na}^+$  and  $\text{Ca}^{2+}$ , whereas



**Figure 2.** Endogenous levels of ions in mitochondria during continued exposure to 25% sea water. (Δ) Calcium. (●) Sodium. (○) Potassium.

K<sup>+</sup> levels showed only minor changes. The Na<sup>+</sup> levels expressed as nmol/mg mitochondrial protein decreased from 1200 to about 600 in 4 days, remains constant at this level till about 8 days and then slowly increased to 800 by about the 14th day. On the other hand, Ca<sup>2+</sup> showed an inverse relationship to Na<sup>+</sup>, rising sharply from the fresh water value of about 200 to 1200 on the 8th day, and returning to the low level on the 13th day. It should be mentioned at this point that only the total amount of the ions have been measured and no attempt was made to distinguish between soluble ionic state and precipitated salts, especially for calcium.

#### *Na<sup>+</sup>—Ca<sup>2+</sup> interactions*

It has been shown earlier (Crompton *et al.*, 1976; Carafoli *et al.*, 1974) that sodium inhibits the uptake of <sup>45</sup>Ca<sup>2+</sup> by mitochondria and that incubation of calcium loaded mitochondria in a medium containing Na<sup>+</sup>, releases the calcium. We have been able to repeat these experiments with fish muscle mitochondria. The results of an experiment where the <sup>45</sup>Ca<sup>2+</sup> uptake by mitochondria isolated from fresh water fish in the presence of increasing concentrations of Na<sup>+</sup> in the medium showed a linear decrease as the Na<sup>+</sup> concentration in the medium increases from 0 to 2.25% (0 to 0.5 M). The <sup>45</sup>Ca<sup>2+</sup> uptake decreases about 6-fold. In another series of experiments, we have observed the total efflux of <sup>45</sup>Ca<sup>2+</sup> from calcium loaded mitochondria, within 5 min of incubation in a Na<sup>+</sup> containing medium (results not given). Crompton *et al.* (1978) have shown a sigmoidal efflux of Ca<sup>2+</sup> in the presence of Na<sup>+</sup>. The reasons for the differences between these results and the present studies need further investigation.

Based on these observations, the following series of experiments were designed. Fishes were exposed to 25% sea water as before and on the 8th day, the muscle mitochondria were isolated. The respiratory capacity, the respiratory control and ADP/O ratios were determined using the oxygen electrode. An aliquot of the mitochondria was treated with NaCl as described in materials and methods, and the above mentioned parameters were measured. Using a third aliquot, the parameters were measured but with the addition of NaCl (20 mM) to the assay medium. NaCl treatment of the mitochondria (by either method mentioned) restored the ADP/O ratio, respiratory control and dinitrophenol stimulated respiration (table 1). No attempts were made in these experiments to ascertain if any stoichiometric relationships were existent.

In another set of experiments, mitochondria from freshwater fishes were loaded maximally with Ca<sup>2+</sup> (Hannah Sulochana *et al.*, 1977b) centrifuged, quickly washed and suspended in the polarographic assay medium. These mitochondria showed decreased ADP/O ratios and respiratory control. Further they responded to NaCl treatment as shown by the restoration of these activities (table 1).

#### *Swelling patterns of mitochondria*

The osmotic swelling and energy dependant contraction of mitochondria were next studied.

(i) *Response of freshwater mitochondria to in vitro changes in osmolarity:* It was found that in 0.25 M sucrose medium muscle mitochondria isolated from fresh water fishes gave fairly reproducible and steady swelling rate. Lower concentrations gave

varying results while at higher concentrations (upto 0.45 M sucrose) the rate was low. In all further experiments 0.25 M sucrose was used as the medium.

(ii) *Passive swelling rate during exposure to salinity:* Upon exposure to stress condition during the first 4 days, mitochondria showed a slight increase in the swelling rate (table 2). But remarkably between the 7th and 9th days, there was a

**Table 2.** Passive swelling rate of mitochondria.

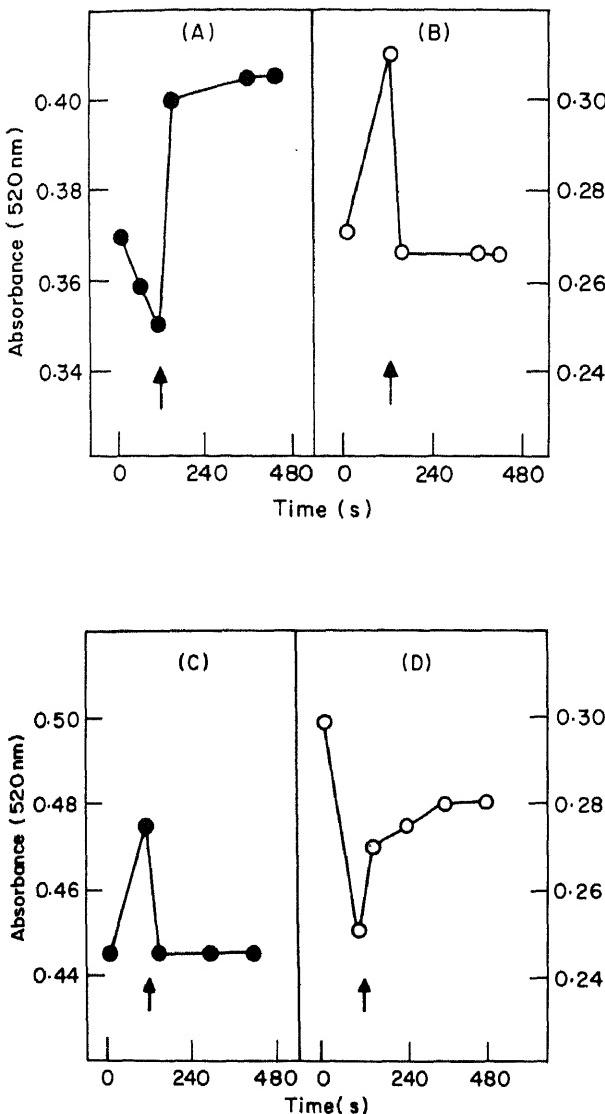
Days after exposure to 25% sea water	Absorbance change (520 nm/min)
0	-0.1
2	-0.15
4	-0.15
7	+0.20
8	+0.15
9	+0.05
10	0
14	-0.20
21	-0.25

— value indicates swelling and  
+ value indicates contraction.

contraction of the mitochondria rather than swelling. On longer exposure, the swelling properties regained their normal swelling response. This indicates a significant change in the internal osmotic environment of the muscle mitochondria during the initial phase of exposure to stress. Interestingly the rate of swelling of these mitochondria in 0.025 M sucrose media showed a very slight increase thereafter remaining constant (data not shown).

### (iii) *Contraction of muscle mitochondria*

The addition of ATP—Mg<sup>2+</sup> mixture to osmotically-swollen mitochondria instantaneously reverses the swelling and induces contraction. This response is analogous to that observed for rat liver mitochondria (Lehninger, 1959). Figure 3A shows a typical pattern of the absorbance changes during the initial swelling and the contraction following the addition of ATP-Mg<sup>2+</sup>, by normal fresh water mitochondria. The extent of the reversal of swelling depends on the ratio of ATP/Mg<sup>2+</sup> added. In the case of freshwater muscle mitochondria, it was seen that a ratio of >1 induced further swelling, whereas ratios of >1 induced contraction. A ratio 1:1 of ATP and Mg<sup>2+</sup> (5 mM each) gave optimal and reproducible results. Moreover, the contraction process was sensitive to bongkekric acid (95% inhibition at 10  $\mu$  M) and oligomycin (50-55%) inhibition at 10  $\mu$  M.



**Figure 3.** Patterns of mitochondrial swelling in 0.25 M sucrose and effect of addition of ATP  $Mg^{2+}$  mixture during continued exposure to 25% sea water.  
 5 mM ATP and 5 mM  $MgCl_2$  were added at the time indicated (↑).  
 (A) Fresh water. (B) 7th day in 25% sea water. (C) 8th day in 25% sea water. (D) 20th day in 25% sea water.

The ATP induced swelling and its reversal by  $Mg^{2+}$  observed in these mitochondria, unlike rat liver mitochondria but similar to that reported by Dow *et al.* (1976)

for muscle mitochondria, was indeed interesting. Table 3 shows the rate of absorbance

**Table 3.** Mitochondrial contraction pattern during long term exposure to 25% sea water.

Days in sea water	Absorbance change at 520 nm on addition of ATP/Mg <sup>2+</sup> mixture	Absorbance change at 520 nm on addition of ATP	Absorbance change at 520 nm on subsequent addition of Mg <sup>2+</sup>
0 (fresh water control)	+0.05	0.086	0.09
7	-0.05	0.19	0.008
14	+0.03	0.12	0.07
20	+0.02	0.096	0.048
24	+0.03	0.08	0.086

ATP and Mg<sup>2+</sup> were added at a concentration of 5 mM each.

changes on the addition of 5 mM ATP, followed by 5 mM Mg<sup>2+</sup> to mitochondria after osmotic swelling in 0.25 M sucrose. The changes on the addition of the mixture of ATP/Mg<sup>2+</sup> (5 mM each) to the mitochondria swollen in the same medium is also given. It is clear from the data presented that the ATP-induced swelling is completely reversed by Mg<sup>2+</sup>. The addition of ATP/Mg<sup>2+</sup> mixture brings about an instantaneous contraction.

#### *(iv) Mitochondrial contraction pattern during long time exposure to salinity stress*

Figure 3 shows the pattern of osmotic swelling in 0.25 M sucrose medium and the effect of addition of ATP/Mg<sup>2+</sup> in mitochondria of fishes from the 7th, 8th and 20th days of exposure to 25% sea water stress. As mentioned earlier, the swelling pattern is significantly altered around the 8th day of stress and these preparations showed contraction in 0.25 M sucrose. It can be seen from the figure that the addition of ATP/Mg<sup>2+</sup> to these contracted mitochondria induced an instant sharp decrease in absorbance, or swelling after which there was no further change in absorbance. The pattern of swelling and contraction of mitochondria from fishes exposed to 25% sea water stress for 20 days showed a reversal of this situation and the pattern resembled that of fresh water mitochondria.

The absorbance changes induced by ATP addition and the reversal of these by Mg<sup>2+</sup> was also checked in mitochondria from the fishes under stress conditions. As is seen in table 3, the ATP induced swelling of these mitochondria increases until the eighth day, whereas there is a decrease in the ability of Mg<sup>2+</sup> to reverse this function. The results indicated an increasing insensitivity of the mitochondria towards Mg<sup>2+</sup> until 8-10 days of exposure to the stress.

The sensitivity of the process to oligomycin also reflected the same changes. However, the mitochondria was inhibited (90-95%) by bongkekric acid.

The results described above strongly indicated a change in the osmotic status of the mitochondria during initial stages of exposure to stress. The drastic reversal of the normal swelling pattern in 0.25 M sucrose observed in the mitochondria from samples on 7th to 9th day of exposure to stress indicated a swollen state of the mitochondria.

## Discussion

### *On the various phases of mitochondrial response*

On exposure of fresh water fish to a higher salinity (iono-osmotic stress) drastic deviations occurred in the functions of the mitochondria isolated from the muscle tissue leading to non-steady states. Two phases of this non-steady state could be clearly distinguished from the pattern of various mitochondrial functions. An initial response phase lasting for about 8 days (under the conditions of stress used), was characterized by changes in mitochondrial functions. These changes involved a loss in mitochondrial phosphorylation capacity, alterations in swelling and contraction, changes in endogenous levels of ions and *in vitro*  $\text{Ca}^{2+}$  uptake capacity as shown earlier (Hannah Sulochana *et al.*, 1977b). This phase is a function of the stress. All these deviations could be elicited earlier by intensifying the stress.

A second (regulatory phase occurs during which mitochondria apparently regain their original status and all the parameters measured reverted back to normal levels. It seemed likely that during this second phase of adaptation, physiological mechanisms like hormonal interactions were probably involved and the cellular environment was also brought back to normal.

### *On the significance of calcium loading*

In recent years, there has been considerable interest on the role of cellular calcium and its physiological relevance (Blum and Hoffmann, 1972; Carafoli and Crompton, 1978). Mitochondria have been envisaged as sinks for intracellular calcium and thus have a vital role in regulating cytosolic calcium concentration. Bygrave (1976a) hypothesized that  $\text{Ca}^{2+}$  by inhibiting the transport of ADP into mitochondria was in a position to modify the energy metabolism of ascites tumour cells. Changes in calcium functions of mitochondria were observed under certain pathological conditions like dystrophy (Mezon *et al.*, 1974). In carbon tetrachloride and uranium nitrate intoxication (Carafoli *et al.*, 1971), physiological significance was attributed to these processes.

Preliminary experiments in this laboratory have shown that soon after exposure, the blood and tissue ionic levels particularly calcium increase (Indu Bashyam, unpublished results). Obviously to maintain intracellular homeostasis, mitochondria accumulate calcium.

Several observations on the mitochondrial behaviour reported by us could be explained by this hypothesis:

- (i) The decreased ADP/O levels, respiratory control ratios, uncoupler stimulated respiration and ATPase levels could be due to the loaded-calcium. Uncoupling effect of calcium was also reported by other workers (Rossi and Lehninger, 1964; Hackenbrock and Caplan, 1969; Jacobus and Brierly, 1969; Thorne and Bygrave, 1974a,b; Bygrave, 1976a).
- (ii) Influx of calcium can cause swelling (Rossi and Lehninger, 1964; Hackenbrock and Caplan, 1969). However, it should be mentioned that the swelling of the organelle under our experimental conditions could also be due to the hyperosmotic intracellular environment. It was suggested that the two opposing forces of electrophoretic cation uptake with osmotic swelling and exchange dependent

cation extrusion and contraction might represent a volume control mechanism for the mitochondrion within the cell which permitted the organelle to maintain its functional integrity in the presence of changes in the ionic environment (Borle, 1973).

Blair (1977) reported that  $\text{Ca}^{2+}$  loaded mitochondria did not show contraction responses to the addition of ATP and  $\text{Mg}^{2+}$ , which is very similar to the observations reported here. This could be either due to the uncoupled nature of the mitochondria, or competition between  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  for the sensitive sites.  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  have been shown to be competitive inhibitors for several ATPases and other enzymes (Lew, 1971; Blum and Hoffmann, 1972; Hackenbrock and Caplan, 1969; Bygrave, 1978; Mason and Tobe, 1977).

(iii) The decreased  $^{45}\text{Ca}^{2+}$  uptake by calcium loaded mitochondria was demonstrated by Carafoli *et al.* (1971). This explains our earlier observation that mitochondria isolated from fish under stress had lowered capacity for  $\text{Ca}^{2+}$  uptake.

#### *On the inter-relationship between sodium and calcium*

Data given in figure 2 on the endogenous levels of ions, shows an inverse relationship between sodium and calcium. As calcium influxed into the mitochondria, sodium was effluxed out. It is interesting that similar effects have been observed under *in vitro* conditions also (table 1). Incubation of calcium-loaded mitochondria with sodium released the inhibited mitochondrial functions.

Carrier mediated exchange between  $\text{Na}^+$  and  $\text{Ca}^{2+}$  is well documented in plasma membrane (Reuter, 1974), mitochondria from cardiac tissue, adrenal cortex and brain (Crompton *et al.*, 1976). In these tissues sodium induced a rapid efflux of  $\text{Ca}^{2+}$  ions. The recent results of Crompton *et al.* (1978) throw some doubt as to whether this recycling is present in skeletal muscle mitochondria. Although critical, quantitative studies are being carried out currently, the results presented in this paper show that muscle tissue may also possess such an  $\text{Na}^+ - \text{Ca}^{2+}$  antiport system.

#### *On the changes in membrane composition*

In our earlier study (Hannah Sulochana *et al.*, 1977b) we had presented physico-chemical evidence to suggest possible conformational changes in mitochondria. Recent analyses of the membrane composition show decreased cholesterol levels in the mitochondria under stressed conditions, conditions which also facilitate swelling of the organelle (Indu Bashyam, unpublished results). Decreased sterol levels could also affect the  $\text{Mg}^{2+}$ -ATPase activity (Graham and Green, 1970; Dianzani *et al.*, 1973; Feo *et al.*, 1975; Astin and Haslam, 1977).

#### *On the reversal of the process or adaptation*

As has been mentioned, the various functions which were inhibited on the 8-9th day of stress were resorted to normal levels on continued exposure to stress by about the 21st day. At present, we do not have satisfactory explanations to present except to say that other physiological mechanisms (particularly hormonal) may have a role to play. Preliminary studies on this aspect with the gill tissue (Suresh Narayan, unpublished data) suggest the possibility that mitochondrialogenesis may be occurring during this adaptive phase. This is under further investigation.

In analogy with the hypothesis of Rasmussen (1975) the presence of an ionic net in the cell that propagates and amplifies the original signal perceived by a membrane receptor, it can be proposed that each ionic compartment maintains its ionic composition at the expense of energy.

### Acknowledgement

This research work was supported by a grant from the University Grants Commission, New Delhi.

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## The role of free amino groups of peptides and proteins in the Folin-Lowry and biuret methods

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MS received 22 February 1980; revised 10 May 1980

**Abstract.** On an equal weight basis polymyxin B and EM 49 which do not contain tyrosine or tryptophan yielded the same colour intensity as proteins in the Folin-Lowry and biuret methods. But, in the absence of reagent C (alkaline copper reagent) polymyxin B and EM 49 yielded no colour in the Folin-Lowry method. Mono-, di- and tri-formyl polymyxins B formed identical amounts of coloured complexes as polymyxin B in the two methods. However, the tetra- and penta-formyl polymyxins B yielded only one-fifth and one-sixth, respectively, of the expected colour in the Folin-Lowry method. Similarly, 40% and 30%, respectively, of the anticipated amount of colour is formed in the biuret method. Formylated and methylated lysozyme and bovine serum albumins form only 70-75% of the expected colour in the Folin-Lowry method. Since formation of colour by reduction of Folin reagent, in the Folin-Lowry method, is at least partly due to complexes of copper, it was inferred that polymyxin B as well as its mono-, di- and tri-formyl derivatives on the one hand and the tetra- and penta-formyl derivatives on the other differ in their ability to complex Cu(II). The former group of compounds was indeed found to complex as many as three Cu(II) ions whereas the tetra- and penta-formyl polymyxins B complexed only one equivalent, under conditions of excess Cu(II). Under conditions of low Cu(II), polymyxin B and all its derivatives complexed only one Cu(II). In proteins, sites other than amino groups which complex Cu(II) probably play a major role in the reduction of the Folin reagent, since methylated lysozyme and bovine serum albumin yield 70-75% of the colour formed by the unmodified proteins in the Folin-Lowry reaction.

**Keywords.** Polymyxin B; acylation of polypeptides; reductive methylation of proteins; Folin-Lowry and Biuret methods; copper chelate of polymyxin B; formyl polymyxins B-Cu(II) chelates; Gramicidin S.

### Introduction

The Folin-Lowry method (Lowry *et al.*, 1951) has been widely used for the estimation of proteins owing to its high sensitivity and simplicity. The colour formed in this method has been attributed to the reduction of the Folin reagent by the copper complexes formed by peptides and proteins and the tyrosine and tryptophan residues therein (Layne, 1957). Specific peptide bonds and favourable steric factors (Wu, *et al.*, 1978) have also been invoked to explain colour formation. The less sensitive

Abbreviations: DABA,  $\alpha$ ,  $\gamma$ -diaminobutyric acid; Thr, threonine; Phe, phenylalanine; Leu, leucine; Val-valine; Orn, ornithine; Pro, proline. Unless otherwise mentioned configuration of amino acids is of the L-type.

biuret method (Gornall *et al.*, 1949) also finds use in the estimation of proteins and the colour formed in this method is the result of copper complexation with proteins. A variety of substances are known to interfere in the Folin-Lowry (Zondag and Boetzlaer, 1960; Diamont *et al.*, 1967; Reider, 1961; Vallejo and Lagunas, 1970; Chou and Goldstein, 1960; Ramachandran and Fraenkel-Conrat, 1958) and biuret (Parvin *et al.*, 1965; Pandey *et al.*, 1961) reactions. Polymyxin B is a basic peptide antibiotic which can be determined by the Folin-Lowry and biuret methods. However, some of the formyl polymyxins are not as chromogenic as polymyxin B. Difficulties encountered earlier (Srinivasa and Ramachandran, 1979) in the determination of formyl derivatives of polymyxin B had been avoided by deformylation of the derivatives prior to analysis.

We present in this paper data on the role of free amino groups of polymyxin B in complex formation with copper ions and the importance of such complexes in the formation of colour in the Folin-Lowry method. The role of free amino groups of proteins like bovine serum albumin and lysozyme in the reduction of Folin-Lowry reagent is also discussed based on data on chromogenicity of methylated and formylated derivatives of the two proteins.

### Materials and methods

Polymyxin B sulphate with biological activity of 7531 IU/mg (Calbiochem, Richmond, California, USA) was a gift from Dr. B. Witkop. Bovine serum albumin and lysozyme were commercial samples from Sigma Chemical Co., St. Louis, Missouri, USA. EM 49 was a gift from Squibb Institute for Medical Research, Princeton, New Jersey, USA. All other chemicals and reagents were from different commercial sources. Folin reagent (Folin and Ciocalteu, 1927) was prepared in the laboratory.

Amino groups were estimated by the ninhydrin method (Rosen, 1957). All colorimetric measurements were carried out using a Junior Coleman Spectrophotometer. A Toshniwal Spectrophotometer Type RL 02 or a Beckman DB Spectrophotometer was used for spectrophotometric measurements.

Partially formylated polymyxin B (PF-polymyxin B) was prepared as described earlier (Srinivasa and Ramachandran, 1978). The crude samples of PF-polymyxin B used had an average degree of substitution of 2.8 groups per mole. Characterized samples with discrete degrees of formylation were prepared as described earlier (Srinivasa and Ramachandran, 1979).

The experimental conditions used for the colour development in the two methods are the same as recommended in the original methods. While solutions of mono, di-, tri- and tetra-formyl polymyxins B were prepared in 0.1 M sodium acetate, penta-formyl polymyxin B was dissolved in aqueous methanol (80%).

### Determination of copper complex formation by polymyxin B and its derivatives

The ability of polymyxin B and its formyl derivatives to complex with copper was determined by the modified continuous variation method (Vosburgh and Cooper, 1941), as follows. To 0.1 ml of polymyxin B sulphate (formyl polymyxin B) solution (0.3 mM), was added 0.025, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 ml of copper sulphate solution (0.3 mM) containing sodium potassium tartrate (0.015%) to obtain molar ratios of 1:0.25; 1:0.5; 1:1; 1:2; 1:3; 1:4 and 1:5 of polymyxins B sulphate to copper sulphate. The volume was made up to 3 ml, with 0.1 N NaOH. After 30 min of mixing, the absorbance was measured at 280 nm. The difference ( $\Delta$ ) between the observed

absorbance and that obtained in the absence of polymyxin B was plotted against the ratio of the concentration of Cu(II) to total concentrations of Cu(II) and polymyxin B. The maximum in the graph indicated the molar ratio of the metal ion to ligand in the chelate formed. Similarly the chelating ability of polymyxin B or its formyl derivatives under a fixed limiting concentration of Cu(II) was determined by adding to 0.1 ml of copper sulphate solution (0.3 mM), 0.025, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 ml of polymyxin B sulphate (formyl polymyxins B) solution (0.3 mM).

#### *Formylation of bovine serum albumin and lysozyme*

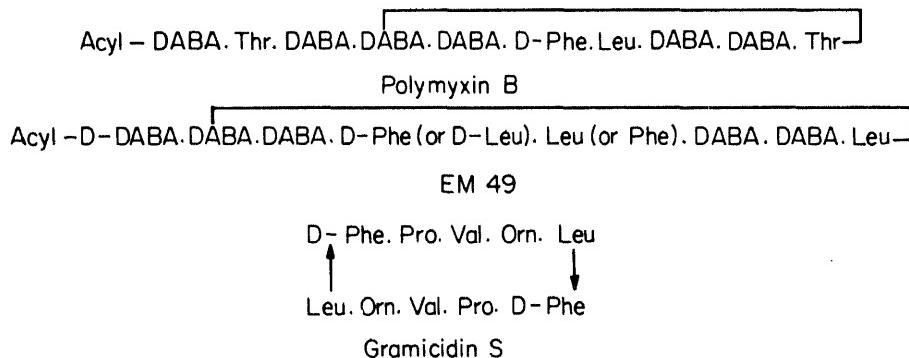
Bovine serum albumin (25 mg) was formylated (Sheehan and Yang, 1958) for 24 h at room temperature. Formylated bovine serum albumin thus obtained had 20 (of the original 62) amino groups still free. Reformylation of this derivative or formylation of bovine serum albumin at 50°C afforded derivatives still containing 15 free amino groups per mol. Masking of all amino groups in bovine serum albumin was not achieved. Formylated lysozyme was prepared similarly and found to contain 0.1 free amino group per mol.

#### *Reductive methylation of bovine serum albumin*

Bovine serum albumin dissolved in 1 ml of 0.25 M borax (pH 9.4) was methylated overnight (Means and Feeney, 1968) using formaldehyde (0.02 ml, 37-40%) and sodium borohydride (4 mg) in the cold overnight. The methylation of all amino groups was rendered possible on addition of additional amounts of sodium borohydride (4 mg) and formaldehyde (0.02 ml) and allowing the reaction mixture to stand in the cold for 6 h longer. The methylated bovine serum albumin showed a content of 0.5 free amino group per mol. Methylated lysozyme was prepared under the above experimental conditions and found to contain 0.9 free amino groups per mol.

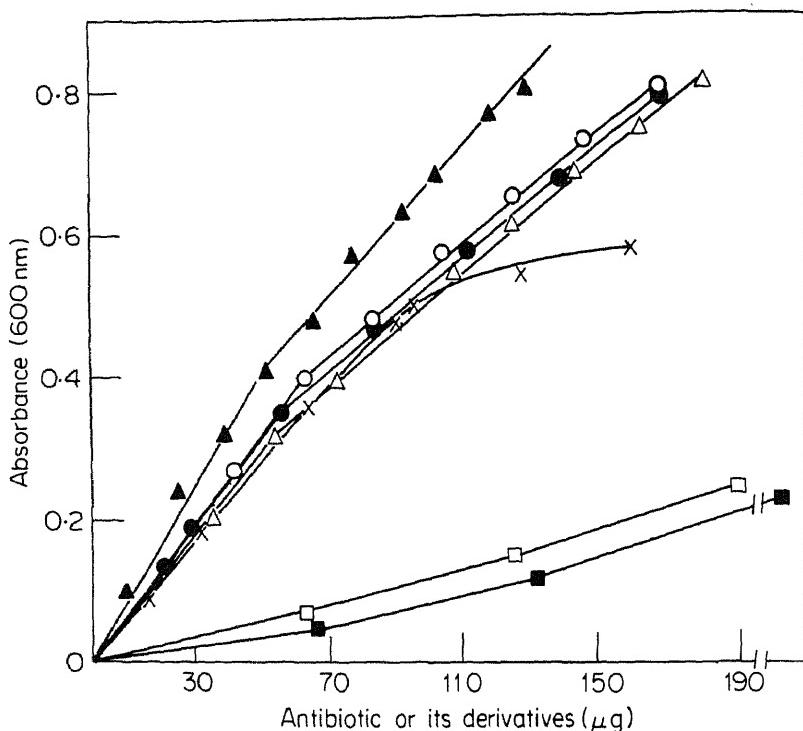
#### **Results and discussion**

On an equal weight basis, polymyxin B and EM 49 (figure 1) which contained neither tyrosine nor tryptophan formed as much, or a little more, colour as lysozyme



**Figure 1.** Structures of cyclic peptides.

in the Folin-Lowry and biuret methods (figures 2 and 3). Bovine serum albumin is

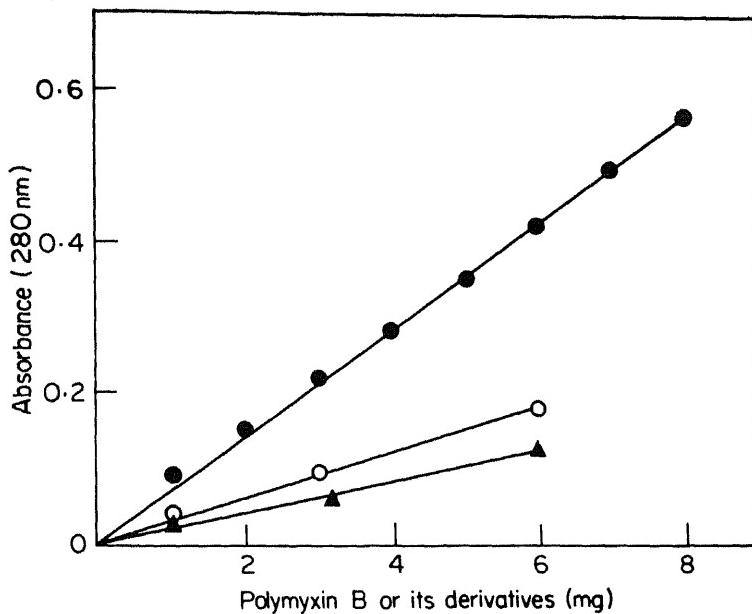


**Figure 2.** Chromogenicity of EM 49, polymyxin B and formyl derivatives of polymyxin B in the Folin-Lowry method.

EM 49, ▲; Polymyxin B, ○; monoformyl polymyxin B, ●; diformyl polymyxin B, △; trifomyl polymyxin B, X; tetraformyl polymyxin B, □; penta formyl polymyxin B, ■.

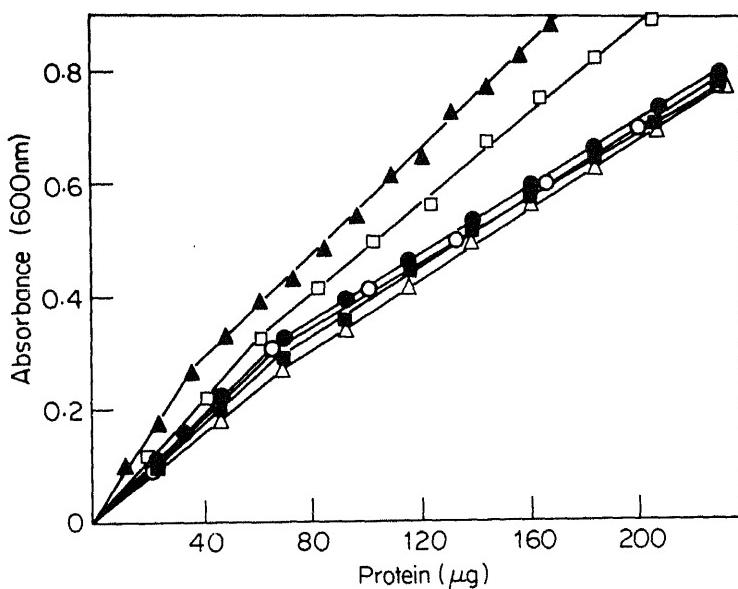
16% less chromogenic (figure 4) than polymyxin B and lysozyme in the Folin-Lowry method. Unlike proteins, polymyxin B and EM 49 formed little or no colour with the Folin reagent in the absence of alkaline copper reagent (reagent C). It is evident from this that the colour formed by polymyxin B and EM 49 in the Folin-Lowry method is due to the reduction of Folin reagent by the copper-complexes of polymyxin B or EM 49 formed on reaction with reagent C. Conversely, the cyclic peptide antibiotic gramicidin S (figure 1) having two free side chain amino groups of ornithine does not reduce Folin reagent either in the presence or absence of alkaline copper reagent (reagent C) in the Folin-Lowry method. This must necessarily mean that compositional and structural factors may play a significant role in the formation of colour in the Folin-Lowry method.

Crude formyl polymyxin B (a mixture of formyl polymyxins B) containing 2.2 free amino groups per mol formed only 75% of the colour given by polymyxin B. However, the mono-, di- and tri-formyl polymyxins B were as efficient as polymyxin B in forming the chromogen. In contrast, the tetra- and penta-formyl polymyxins B



**Figure 3.** Comparison of the colour formed by polymyxin B and its formyl derivatives in the biuret method.

Polymyxin B, mono-, di- and tri-formyl polymyxins B, ●; tetraformyl polymyxin B, ○; pentaformyl polymyxin B, ▲.



**Figure 4.** Colour concentration relationships for bovine serum albumin, lysozyme and their derivatives.

Lysozyme, ▲; bovine serum albumin, □; formylated lysozyme, ○; methylated lysozyme, ●; formylated bovine serum albumin, ■; methylated bovine serum albumin, Δ.

yielded one-fifth and one-sixth, respectively, of the colour expected. Unlike polymyxin B or its formyl derivatives with lower degree of substitution, the tetra- and penta-formyl derivatives formed only 40% and 30%, respectively, of the expected colour in the biuret method (figure 3).

It, therefore, appears from the above data that the colour formed in the Folin-Lowry and biuret methods by polymyxin B is largely due to amino groups and their ability to complex Cu(II). In those cases of peptides or proteins containing neither tyrosine nor tryptophan the overwhelming role of amino groups or the sites that can bind copper ions and of characteristic Cu(II) complexes formed by them in the formation of colour through reduction of the Folin reagent is easily recognised. A study (Berg, 1971) in which the N-acetyl derivatives of D-glucosamine and D-galactosamine were found to form only 20% as much colour as the corresponding free amino sugar points to the importance of amino groups in the reaction given by amino sugars.

The modified continuous variation method of Job (Vosburgh and Cooper, 1941) revealed the correctness of the inference about the importance of amino groups since polymyxin B and mono-, di- and tri-formyl polymyxins B were found to complex with three Cu(II) under conditions of Cu(II) excess, while the tetra- and penta-formyl polymyxins B complexed only one (figure 5). However, when Cu(II) was limiting polymyxin B and all its formyl derivatives complexed only one Cu(II)

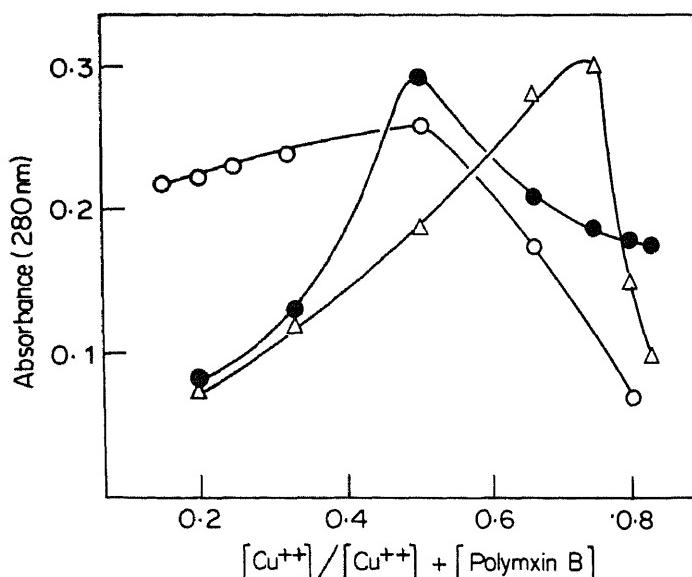


Figure 5. Modified job plot for complex formation of Cu(II) by polymyxin B and formyl polymyxins B.

The chelating ability of polymyxin B and its formyl derivatives under limiting concentration of Cu(II), ●; the chelating ability of tetra and pentaformyl polymyxin B under concentrations of Cu(II) excess, ○; copper complexing ability of polymyxin B and mono-, di-, and trifomyl polymyxins B under concentrations of Cu(II) excess, Δ.

(figure 5). The actual conditions of colour formation during routine analysis are such that the ratio of Cu(II): polymyxin B is always greater than 4:1. An earlier

investigation (Brintzinger, 1960) had noted the ability of polymyxin B sulphate to form 1:1 and 1:2 complexes with copper (II), at lower pHs than is realised in the experiments mentioned earlier.

Tyrosine, tryptophan and -SH groups of proteins are known to reduce Folin reagent even in the absence of alkaline copper reagent (reagent C). The colour formed is however enhanced three- to four-fold by reagent C. Modified bovine serum albumin and lysozyme which contain no free amino groups form almost 70-75% of the expected colour (figure 4). Thus, 25-30% of the colour formed in the method as normally used is assignable to free amino groups of the two proteins. It is clear that in such proteins additional sites which can complex Cu(II) possibly play an even greater role relative to free amino groups in colour formation. On the other hand, the hexosaminyI and sialyl residues in the oligosaccharide chains of the Armadillo salivary gland glycoprotein have been shown to exert a marked shielding effect in formation of colour by the protein in the Folin-Lowry reaction (Wu *et al.*, 1978). This glycoprotein contains only non-chromogenic amino acids—glycine, threonine, serine, glutamic acid, alanine and valine, and its higher reactivity with the Folin-Lowry reagent is attributed to both acid-sensitive and acid-resistant peptide linkages involving threonine and valine, since these two amino acids account for two-thirds of the total amino acids in the protein. The absence of proline and cysteine in this protein is considered as favouring better complex formation with copper ions. Therefore, it is to be concluded that compositional, structural and steric factors affect colour formation by peptides and proteins in the Folin-Lowry method. In the biuret reaction, methylation or formylation of the two proteins studied was not found to alter the chromogenicity.

All but the tetra- and penta-formyl polymyxins B are known to be fully biologically active (Srinivasa and Ramachandran, 1978). It is possible that the amino groups involved in complex formation with the extra copper ions under conditions of Cu(II) excess are the two side chain amino groups of residues 1 and 3 (Srinivasa and Ramachandran, 1980) involved in the biological action of triformyl polymyxin B. That some charged/uncharged (Feingold *et al.*, 1974) amino groups of polymyxin B may interact with the acidic phospholipids to complex with the divalent cations such as  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (Newton, 1955) which normally stabilise (Lieve, 1974) the outer membrane of Gram negative bacteria to destabilize its recognized. It is conceivable that those amino groups of polymyxin B involved in this activity and in the binding of excess Cu(II) encountered in the 3:1 complexes are the same.

### Acknowledgement

One of the authors (B.R.S.) wishes to thank the Council of Scientific and Industrial Research, New Delhi for the award of a fellowship.

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## **On the regulation of L-hydroxyproline dissimilatory pathway in *Pseudomonas aeruginosa* PAO**

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MS received 30 June 1979; revised 29 April 1980

**Abstract.** The enzymes involved in the regulation of L-hydroxyproline degradation in *Pseudomonas aeruginosa* PAO were investigated. L-hydroxyproline when present in the growth medium induces all the four enzymes in the pathway. Growth of the cells in L-proline also weakly induced the enzymes. The organism failed to utilize D-allo-hydroxyproline due to permeability factors. Mutants blocked in the oxidative pathway of L-hydroxyproline were isolated and enzymatically characterized. In all the mutants lacking any one of enzymes of the metabolic pathway, L-hydroxyproline is still active in inducing the remaining enzymes of the pathway suggesting that L-hydroxyproline has intrinsic inducer activity.

**Keywords.** Metabolism of L-hydroxyproline; regulation; mutants; *Pseudomonas aeruginosa* PAO.

### **Introduction**

Many species of *Pseudomonas* utilize a variety of amino acids as the sole source of carbon and nitrogen (Ornston, 1971). L-Hydroxyproline (Hyp), a constituent imino acid of connective tissue material of animals has been reported to be toxic to several microorganisms. However, three bacteria catabolize Hyp (Adams, 1970). The studies of Adams and his colleagues (Adams, 1970) and Jayaraman and Radhakrishnan (1965a, b) have provided detailed evidence for the degradation of Hyp.

It has been previously shown that all the four enzymes of Hyp degradative pathway, in *P. putida* (Gryder and Adams, 1969), *Pseudomonas* I (Jayaraman and Radhakrishnan, 1965a; Manoharan, unpublished data) and *Achromobacter* (Jayaraman and Radhakrishnan, 1965b), are inducible. These biochemical studies need to be augmented by genetic analysis to establish whether the nature of induction of the enzymes in the pathway could be correlated with the arrangement of genes. Since several mapping techniques are available in *P. aeruginosa* PAO (Holloway *et al.*, 1971), it was decided to investigate the regulation of enzymes of Hyp pathway in

**Abbreviations:** Hyp: L-hydroxyproline; D-allo-Hyp: D-allo-hydroxyproline; Pro: L-proline; Lys: L-lysine; Glu: L-glutamic acid; Ala: L-alanine; Val: L-valine; PCHA: 3,  $\Delta^1$  pyrroline-4-hydroxy-2-carboxylate, pyrroline-hydroxycarboxylate; KGSA:  $\alpha$ -ketoglutarate semialdehyde.

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this organism in an effort to correlate the control mechanisms and gene arrangement. In this paper, some of the properties of the inducible enzyme system of *P. aeruginosa* PAO that degrades Hyp and biochemical characterization of *Hyp*<sup>-</sup> mutants are reported. Detailed genetic mapping studies of *Hyp* region of the chromosome have been presented elsewhere (Manoharan and Jayaraman, 1979).

### Materials and methods

#### Bacterial strains, media and growth conditions

The strains used in this study were derived from *P. aeruginosa* PAO (ATCC 15692) and are listed in table 1. The composition of minimal salt solution has been described previously (Kunthala and Radhakrishnan, 1964). For the preparation of minimal

Table 1. Strains of *Pseudomonas aeruginosa*

Strain	Genotype	Sex	Origin, source or reference
MI2	<i>Hyp</i> <sup>+</sup> , <i>trp-6</i>	FP <sup>-</sup>	Kageyama (1970)
MI20	<i>Hyp</i> <sup>-</sup> , <i>trp-6</i>	FP <sup>-</sup>	Derived from MI2
MI21	<i>Hyp</i> <sup>-</sup> , <i>trp-6</i>	FP <sup>-</sup>	"
MI22	<i>Hyp</i> <sup>-</sup> , <i>trp-6</i>	FP <sup>-</sup>	"
MI24	<i>Hyp</i> <sup>-</sup> , <i>trp-6</i>	FP <sup>-</sup>	"
MI25	<i>Hyp</i> <sup>-</sup> , <i>trp-6</i>	FP <sup>-</sup>	"
MI26	<i>Hyp</i> <sup>-</sup> , <i>trp-6</i>	FP <sup>-</sup>	"
MI27	<i>Hyp</i> <sup>-</sup> , <i>trp-6</i>	FP <sup>-</sup>	"
PAO67	<i>Hyp</i> <sup>+</sup> , <i>his-67</i>	FP <sup>-</sup>	Stanisich and Holloway (1972)
PAO677	<i>Hyp</i> <sup>-</sup> , <i>his-67</i>	FP <sup>-</sup>	Derived from PAO67
PAO303	<i>Hyp</i> <sup>+</sup> , <i>argB18</i>	FP <sup>-</sup>	Haas <i>et al.</i> (1977)
PAO3031	<i>Hyp</i> <sup>-</sup> , <i>argB18</i>	FP <sup>-</sup>	derived from PAO303
PAO307	<i>Hyp</i> <sup>+</sup> , <i>argC54</i>	FP <sup>-</sup>	Haas <i>et al.</i> (1977)
PAO3071	<i>Hyp</i> <sup>-</sup> , <i>argC54</i>	FP <sup>-</sup>	derived from PAO307
PAO381	<i>Hyp</i> <sup>+</sup> , <i>leu-38</i>	FP <sup>-</sup>	Pemberton and Holloway (1972)
PAO3811	<i>Hyp</i> <sup>-</sup> , <i>leu-38</i>	FP <sup>-</sup>	derived from PAO381
PAO381211	<i>Hyp</i> <sup>-</sup> , <i>leu-38</i>	FP <sup>-</sup>	"

Genotype symbols are the same as those used in *E. coli* (Bachmann, *et al.*, 1976) except that *Hyp*<sup>-</sup> signifies inability to utilize Hyp as a source of carbon and nitrogen.

medium, glucose (or glycerol) and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> were added to the minimal salt solution at a concentration of 20 mM and 10 mM respectively. When amino acids were used as the sole source of carbon and nitrogen, they were added at a concentration of 20 mM. Amino acid supplements to the salt medium were added at a concentration of 1 mM. Growth conditions have already been described (Kunthala and Radhakrishnan, 1964).

#### Measurement of oxygen uptake with whole cells

Cells grown in a desired medium were harvested during the late exponential phase

of growth, washed twice with 50 mM potassium phosphate buffer (pH 7.0) and resuspended in the same buffer to give a turbidity of 600 Klett Units ( $\equiv$  2.5 mg of protein/ml  $\equiv$  8 mg dry weight/ml). Oxygen uptake was measured polarographically as described by Manoharan and Jayaraman (1978a) using a Clark oxygen electrode at 30°C and the rate of O<sub>2</sub> uptake was expressed as nmol of O<sub>2</sub> uptake/min/mg of protein.

#### *Altering the cell permeability by EDTA treatment*

The method used to enhance the permeability of the cells of D-allo-Hyp using EDTA is similar to that described for *Escherichia coli* (Heppel, 1968). Washed cells grown in Hyp were resuspended in 0.12M Tris-HCl buffer (pH 8.2) to a turbidity of 600 Klett Units. Aliquots of cell suspensions (10 ml) were treated with various concentrations of EDTA for 2 min and the cells were collected by centrifuging at 1,000 g for 5 min. The pellets were suspended in the original volume with 50 mM phosphate buffer (pH 7.0) containing chloramphenicol (100  $\mu$ g/ml). The capacity of the cells to utilize D-allo-Hyp was then determined in a reaction mixture (1 ml) containing 5  $\mu$ mol of D-allo-Hyp, 50 mM glycine-NaOH buffer (pH 9.4) and an aliquot of EDTA-treated cell suspension. After 1 h of incubation in a 30°C water bath shaker the product  $\Delta$ -PCHA formed in the reaction, was measured after acid conversion to pyrrole-2-carboxylate by colour formation with p-dimethylaminobenzaldehyde (Radhakrishnan and Meister, 1957; Kunthala and Radhakrishnan, 1964).

#### *Preparation of enzyme extract and assay of enzymes*

The preparation of enzyme extract has been described previously (Manoharan and Jayaraman, 1978a) and is briefly described below:

- (i) Hydroxyproline-2-epimerase was assayed in a 2-step procedure described by Jayaraman and Radhakrishnan (1965b). Activity of the epimerase is expressed as  $\mu$  mol of D-allo-Hyp formed/mg of protein/h and using this method it was not possible to detect the concentration of D-allo-Hyp below 0.01  $\mu$  mol/ml.
- (ii) Allohydroxy-D-proline oxidase was assayed by the method of Jayaraman and Radhakrishnan (1965a). The product of the reaction PCHA was measured after its conversion to pyrrole-2-carboxylate as described earlier in this paper. The enzyme activity is expressed as  $\mu$  mol/ml of pyrroline hydroxy carboxylate formed per mg of protein/h and values below 0.01  $\mu$  mol/ml of pyrroline hydroxy carboxylate could not be determined.
- (iii) The preparation of substrate and assay for pyrroline hydroxy carboxylate deaminase were carried out as described by Adams (1971). The disappearance of the substrate from the reaction mixture after incubation for 1 h is measured by the colorimetric test for pyrrole-2-carboxylate as described earlier in this paper. The activity of the enzyme is expressed as  $\mu$  mol of PCHA mg utilized mg of protein/h and under the assay conditions it is not possible to estimate PCHA concentrations below 0.01  $\mu$  mol/ml.
- (iv)  $\alpha$ -Ketoglutarate semialdehyde was synthesised according to the procedure of Sharma and Blumenthal (1973). The assay of  $\alpha$ -ketoglutarate semialdehyde dehydrogenase is based on the oxidation of KGSA tol2-ketoglutarate as measured by the rate of NADP reduction at 340 nm (Adams and Rosso, 1967). The activity was expressed

as  $\mu$  mol of NADP reduced/min/mg of protein and the reduction below the level of 0.05  $\mu$  mol/ml could not be measured.

#### Amino acid transport assay

The transport of [ $^{14}$ C]-L-proline by the whole cells was assayed as described previously (Manoharan and Jayaraman, 1978a).

Protein was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

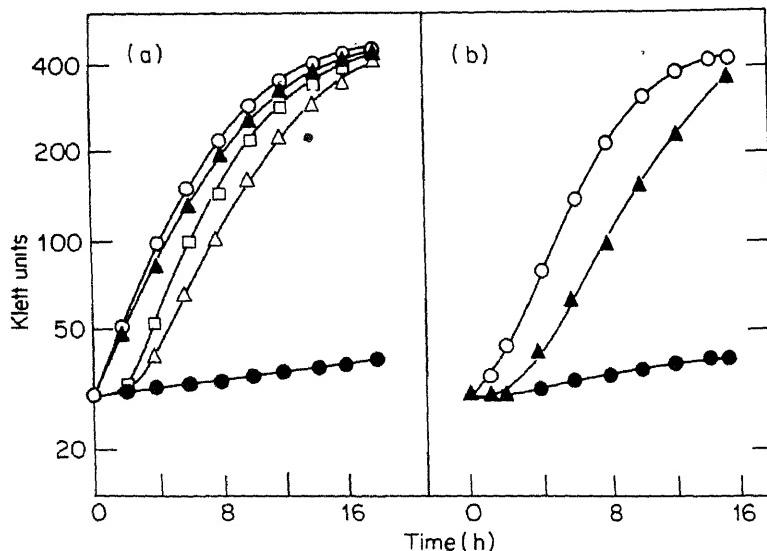
#### Isolation of Hyp mutants

Mutants incapable of utilizing Hyp as a sole source of carbon and nitrogen were isolated after treating the cells with N-methyl-N'-nitro-N-nitroso-guanidine as described previously (Manoharan and Jayaraman, 1978b). The clones which retained the ability to grow on minimal medium agar plate but failed to grow on Hyp agar plate were kept for testing the various blocks in the Hyp pathway. Only stable Hyp<sup>-</sup> mutants (reversion frequency between  $5 \times 10^{-8}$  and  $10^{-9}$ ) were used in this study.

## Results

#### Growth of *P. aeruginosa* on amino acids

Cells of *P. aeruginosa* M12 grown on minimal medium were tested for their ability to grow on a few selected amino acids as sole source of carbon and nitrogen and the kinetics of growth (figure 1A) indicated that in this organism Pro and glutamate are



**Figure 1.** (A) Growth of *P. aeruginosa* M12 on minimal medium, O; Glu, ▲; Pro, □; Hyp, Δ; D-allo-Hyp, ●. Washed cells grown on minimal medium were used as inoculum and growth was followed at an interval of 30 min using a Klett-Summerson colorimeter with yellow-green filter. (B) Influence of prior growth in Hyp or incubation in D-allo-Hyp on the ability of the cells to utilize Hyp, O; Pro, ▲; allo Hyp, ●. Washed cells grown in minimal medium were incubated overnight in D-allo-Hyp medium (300 Klett units). The cells incubated in D-allo-Hyp or grown in Hyp were washed and inoculated into the various media and growth was measured by turbidimetry.

more readily utilized. Growth of the organism with Hyp occurred after a lag period of 3 h and D-allo-Hyp on the contrary, failed to initiate growth even after a prolonged period of incubation (12 h). Attempts to initiate growth of the organism with D-allo-Hyp by changing the cultural conditions were unsuccessful. For example, prior growth of the organism in Hyp (figure 1B) or prolonged incubation of the cells in D-allo-Hyp (figure 1B) could not influence growth with D-allo-Hyp. The inability of the organism to grow at the expense of D-allo-Hyp was unexpected as the only known bacterial route of Hyp oxidation is via D-allo-Hyp (Adams, 1970). It is well known that regardless of enzymic composition, bacterial cells cannot utilize substances that do not permeate through cell membranes and since there are many instances of D-amino acids not permeating freely into bacterial cells (Wild *et al.*, 1978) it could be that cells of *P. aeruginosa* are not permeable to D-allo isomer of Hyp.

#### Oxygen uptake studies

Catabolic utilization of Hyp isomers in *Pseudomonas* is an oxidative process (Adams, 1970; Kuttan and Radhakrishnan, 1973). To find the ability of Hyp, Pro and Glu to induce enzymes of Hyp pathway, cells grown on any one of the amino acids were tested for the presence of the enzymes of Hyp pathway by checking their ability to oxidize Hyp and D-allo-Hyp. The capacity to oxidize Pro in these cells were also tested to determine whether the enzymes of Hyp and Pro degradative pathways are

**Table 2.** Initial rate of oxidation a few amino acids by *P. aeruginosa* M12.

Cells grown in	Oxygen uptake with various substrates (nmol of O <sub>2</sub> uptake/min/mg of protein)			
	Hyp	D-allo-Hyp	Pro	Glu
Minimal medium	—	—	—	—
Glu	—	—	—	—
Pro	23	—	41	41
Hyp	62	42	24	20

The measurement of oxygen uptake was done with the washed cells grown on different media as described in materials and methods. The substrates were used at a concentration of 5 μmol/ml. The figures given in the table were corrected for endogenous rate (usually below 5 nmol/min/mg of protein). — No detectable rate of oxygen uptake. Under the assay conditions, the lower limit of detectable O<sub>2</sub> uptake was 2.4 nmol/ml.

cross inducible by Pro and Hyp respectively. The results presented in table 2 indicated that Hyp oxidation is inducible as the cells grown in minimal medium and Glu were unable to oxidize the Hyp isomers immediately. The proline-grown cells however showed a lower rate of oxidation with Hyp (50% of the induced level of oxidation) and a similar response was observed on adding Pro to the cell grown in Hyp.

As the low rate of Pro oxidation in Hyp-grown cells and Hyp oxidation in Pro grown cells was immediate without a lag phase, it was of interest to know the nature of this oxidation process. Prior incubation of the cells in chloramphenicol failed to abolish the low rate of oxidation process (table 3). Further, incubation of Hyp

**Table 3.** Effect of chloramphenicol on the initial kinetics of oxidation of L-imino acids in the cells of *P. aeruginosa* M12.

Grown in		Oxygen uptake with various substrates (nmol of O <sub>2</sub> uptake/min/mg of protein)		
		Hyp	D-allo-Hyp	Pro
Hyp	Untreated	62	40	24
	Chloramphenicol treated	60	37	22
Pro	Untreated	23	—	41
	Chloramphenicol treated	22	—	44

Washed cells grown in Hyp and Pro were treated with chloramphenicol (100  $\mu$ g/ml) for 10 min prior to the addition of the substrate. The measurement of O<sub>2</sub> uptake was done as described in table 2. — No detectable rate of oxygen uptake.

grown cells in Pro (upto a period of 10 min) and Pro grown cells in Hyp (upto a period of 30 min) could not enhance the oxidation process above this low rate of oxidation (table 4). Thus, it appears that growth in one L-imino acid simultaneously causes a low degree of cross induction of enzymes associated with the oxidation of the other L-imino acid.

**Table 4.** Effect of preincubation on the oxidation of Hyp and Pro in the cells of *P. aeruginosa* M12 grown in Pro and Hyp.

Period of incubation with the substrate (min)	Rate of O <sub>2</sub> uptake (nmol of O <sub>2</sub> uptake/min/mg of protein)	
	Pro oxidation by Hyp grown cells	Hyp oxidation by Pro grown cells
1	23	22
15†	36	23
15‡	42	23

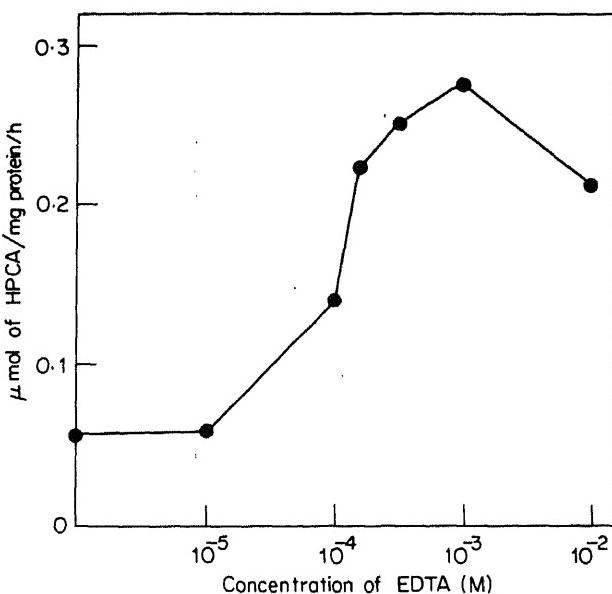
Hyp or Pro was added to the washed cells grown in Pro or Hyp respectively. Samples of cells suspension (3 ml) were taken at 1 and 15 min and the rate of O<sub>2</sub> uptake was measured as described in table 2.

† denotes incubation of cells in 5  $\mu$ mol/ml of Pro or Hyp.

‡ denotes incubation of cells in 10  $\mu$ mol/ml of Pro or Hyp.

### *Effect of EDTA treatment on the oxidation of D-allo-Hyp*

It has been shown that treatment of *E. coli* cells with EDTA remarkably enhances the permeability property and the cells after EDTA treatment transport many organic compounds that are usually excluded from entering into the cells (Leive, 1965). Therefore, attempts were made to find out whether exposing the cells of *P. aeruginosa* to EDTA could influence the utilization of D-allo-Hyp. It can be seen from figure 2 that the ability of L-Hyp grown cells to oxidize D-allo-Hyp became



**Figure 2.** Effect of EDTA treatment on the capacity of Hyp grown cells to oxidize D-allo-Hyp. EDTA treatment and the measurement of D-allo-Hyp oxidation are described in materials and methods.

evident when the cells were treated with 0.1 mM EDTA and the capacity to oxidize D-allo-Hyp rose sharply until the concentration of EDTA reached 1 mM. Thereafter, higher concentrations of EDTA influenced reduction in the rate of D-allo-Hyp oxidation which may indicate possible solubilization of the particulate D-allo-Hyp oxidase. Similarly, treating the cells with toluene (20-50  $\mu$  l) influenced the uptake of D-allo-Hyp (Manoharan and Jayaraman, 1978b). The results suggest that inability of the cells to utilize D-allo-Hyp is a reflection of the absence of transport system/s for D-allo-Hyp in *P. aeruginosa*.

### *Status of enzymes of L-Hyp pathway in the cells grown on different substrates*

In order to assess the sequence of inductive events, specificity and degree of induction of enzymes of Hyp pathway, the specific activities of the four enzymes of the path-

way (figure 3) were determined in the cells grown on a range of substrates. The absence of enzymes of Hyp pathway in cells grown in minimal medium and presence of the enzymes at high levels in Hyp grown cells indicated the inducible nature of these

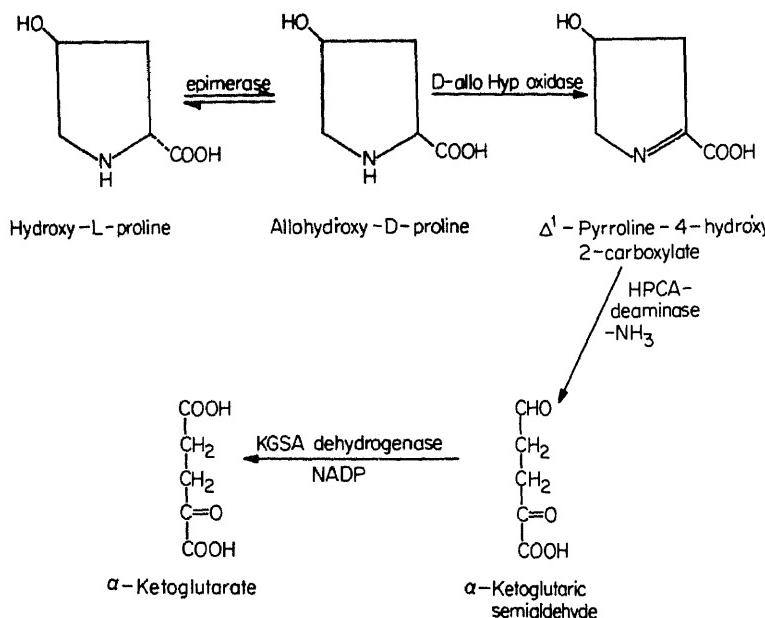


Figure 3. The pathway of Hyp dissimilation in *P. aeruginosa* PAO.

enzymes (table 5). The enzymes were also present at low levels in the cells grown with Pro indicating the occurrence of a low level induction of Hyp pathway upon growth with Pro and this observation explains the low rate of oxygen uptake obtained in these cells upon L-Hyp addition. Cells grown in L-glutamate were found to contain residual levels of D-allo-Hyp oxidase and  $\alpha$ -ketoglutarate semialdehyde dehydrogenase. Presence of these enzymes in glutamate grown cells was unexpected

Table 5. Effect of amino acids on the levels of enzymes of Hyp degradative pathway in *P. aeruginosa* PAO.

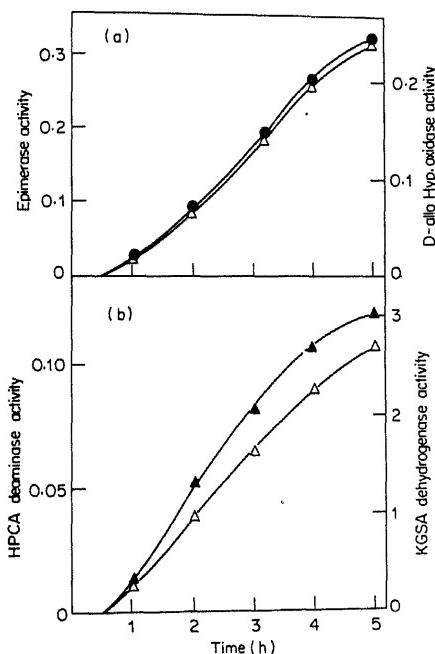
Growth media	Epimerase	D-allo-Hyp oxidase	PHCA deaminase	KGSA dehydrogenase
Minimal medium	—	—	—	—
Glu	—	0.015	—	1.70
DL-Val	—	0.09	—	1.50
Pro	0.055	0.196	0.086	1.77
Hyp	0.41	0.28	0.16	3.55

The activity of the enzyme is determined in the washed cells grown on various media as described in materials and methods. The activity of the enzymes is expressed as described in materials and methods. — No detectable enzyme activity and the lowest limit of detectable values are given in materials and methods.

and it suggested that owing to the interconversion of glutamate  $\rightleftharpoons$  proline, a 'non-specific' D-allo-Hyp oxidase was induced at low level in these cells (Manoharan and Jayaraman, 1978a). The occurrence of isoenzymes of  $\alpha$ -ketoglutarate semialdehyde dehydrogenase has been found in the cells of *P. putida* grown with Hyp and D-glutarate. The 'non-specific' species of  $\alpha$ -ketoglutarate semialdehyde dehydrogenase has been detected in the cells of *P. putida* grown in Lys, Pro and Glu (Adams and Rosso, 1966, 1967; Koo and Adams, 1974).  $\alpha$ -Ketoglutarate semialdehyde dehydrogenase activity was also detected in the cells of *P. aeruginosa* grown on Lys (Fothergill and Guest, 1977). A 'non-specific'  $\alpha$ -ketoglutarate semialdehyde dehydrogenase activity in the cells of *P. aeruginosa* PAO grown on DL-Ala and DL-Val was also observed (unpublished data). However, the possible involvement of the enzyme in the degradation of Glu, Pro, DL-Ala and DL-Val is not clear now.

#### *Induction enzymes of Hyp pathway*

Since the enzymes of the pathway are found to be inducible, it was attempted to follow the time course of appearance of the enzymes in cells grown in minimal medium during induction with Hyp and to determine whether they increased in constant proportion to one another. As shown in figure 4A and 4B there was a



**Figure 4.** Time course of induction of enzymes of Hyp pathway in *P. aeruginosa* M12.

Washed cells grown in minimal medium were suspended in minimal salt solution + trp (1mM) to a turbidity of 300 Klett units. After 15 min incubation at 37°C, L-Hyp (20mM) was added to the culture. At various time intervals, aliquots of cells suspension (50 ml) were withdrawn and harvested by centrifugation. The pellet was washed once with 50 mM phosphate buffer (pH 7.0) containing chloramphenicol (100  $\mu$ g/ml) and resuspended in the same buffer to a turbidity of 600 Klett units. Preparation of enzyme extract and assay of the enzymes are described in a materials and methods.

distinct lag period of 30 ( $\pm 5$ ) min and thereafter there was a gradual increase in the specific activity of each enzyme. However, it was not possible to observe a distinct temporal lag between the appearance of each enzyme of the pathway. Addition of chloroamphenicol at any time between 0-30 min of Hyp addition prevented completely the appearance of the enzymes indicating the inducible nature of the enzymes. In 5 h, all the enzymes of Hyp pathway reached the fully induced levels and similar results were obtained for *Pseudomonas* I (unpublished results) and *P. putida* (Gryder and Adams, 1969).

#### *Other inducers of the pathway*

A few compounds were tested in an attempt to find other inducers of the pathway. They included some non-metabolizable analogues like thioproline, acetylproline and glycylproline. Washed cells of the organism grown in minimal medium were incubated with analogues (20 mM) for 6 h and the sonicated extracts were checked for the presence of the enzymes of the pathway. Absence of Hyp pathway enzymes indicated that none of these compounds is capable of inducing the enzymes. Since the other intermediates of the pathway KGSA and PCHA are unstable and cannot serve as growth substrates. These compounds were not tested as the possible inducer of the pathway.

#### *Permeability status*

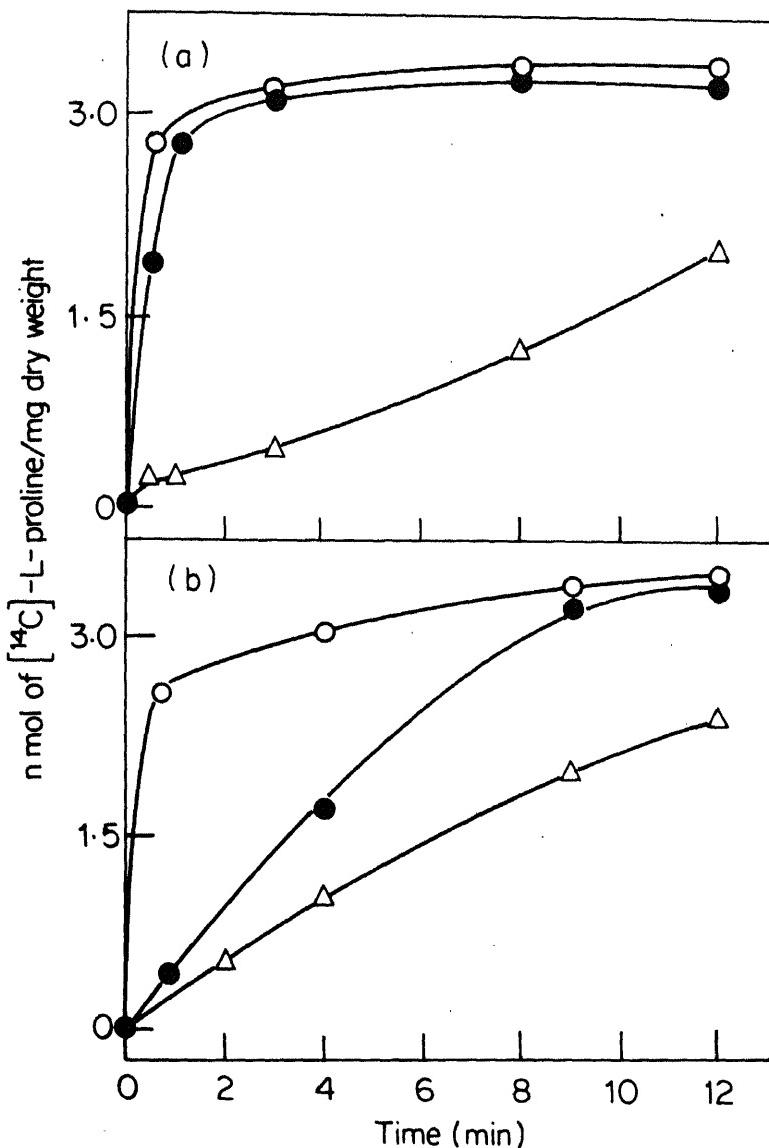
The kinetic study on the uptake of [ $^{14}\text{C}$ ]-L-proline by the cells grown on different carbon sources was undertaken to understand the specificity of L-imino acid transport systems. Both L-Pro and L-Hyp grown cells transported immediately [ $^{14}\text{C}$ ]-L-Pro as opposed to the cells grown on minimal medium and Glu which showed inducible kinetics (figure 5A). Further, addition of unlabelled Hyp inhibited the uptake of [ $^{14}\text{C}$ ]-L-Pro (figure 5B) in the cells grown with Pro and Hyp indicating that the same transport systems could be used by both L-imino acids.

#### *Nature of the genes for the enzymes oxidizing L-Hyp*

Genes specifying several peripheral degradative pathways in *Pseudomonas* are borne on transmissible plasmids (Chakrabarty, 1972; Rheinwald *et al.*, 1973) which can be eliminated from the cells by growth with mitomycin C (Chakrabarty *et al.*, 1973). When individual colonies (4200 numbers) were tested on Hyp agar plates for the spontaneous loss of *Hyp*<sup>+</sup> phenotype, it was not possible to observe *Hyp*<sup>-</sup> clones. Further, growth of the cells with different concentrations of mitomycin C (10-30  $\mu\text{g/ml}$ ) and acriflavin (10-75  $\mu\text{g/ml}$ ) failed to cure the *Hyp*<sup>+</sup> phenotype. The results suggested that genes for the Hyp pathway enzymes are not plasmid derived.

#### *Phenotypic property and characterisation of *Hyp*<sup>-</sup> mutants*

A series of mutants incapable of utilizing Hyp as sole source of carbon and nitrogen were derived from the donor and recipient strains of *P. aeruginosa* after treatment with N-methyl-N'-nitro nitrosoguanidine (table 1). The different *Hyp*<sup>-</sup> mutants were analyzed for the absence of enzymes of Hyp pathway to determine the genetic block. As can be seen from table 6, the epimerase activity was undetectable in the cell extracts of the mutants MI20, MI21, MI24, MI26, MI27, PAO677 and PAO3071.



**Figure 5.** (A) The rate of [<sup>14</sup>C]-proline uptake in the cells of *P. aeruginosa* M12 grown in Pro, O; Hyp, ●; Glu and minimal medium, ▲. (B) Inhibition of [<sup>14</sup>C]-L proline uptake by Hyp in the cells grown with Pro or Hyp; in the absence of Hyp (O) in presence of 0.5 μmol/ml, (●); and 1 μmol/ml (▲) L-Hyp.

Washed cells grown on different media were resuspended in minimal salt solution (0.2 mg protein/ml = 0.8 mg dry weight/ml) and incubated with [<sup>14</sup>C]-L-proline. The final concentration of [<sup>14</sup>C]-L-proline was 5 nmol/ml (= 5 × 10<sup>4</sup> cpm/ml). At various time intervals, 1 ml of cells suspension was filtered through a membrane filter (0.45 μ) and washed with 10 ml of minimal salt solution. The radioactivity was determined in a liquid scintillation spectrometer.

Though these mutants lacked epimerase, presence of other enzymes of the pathway suggested normal functioning of Hyp transport system in these mutants and hence they were assigned to the class of epimerase<sup>-</sup> mutants.

**Table 6.** Levels of enzymes of Hyp degradative pathway in the *Hyp*<sup>-</sup> mutants of *aeruginosa* PAO.

Strain	Genotype	Epimerase	D-allo-Hyp oxidase	PCHA deaminase	KGSA dehydrogenase
MI2	<i>Hyp</i> <sup>+</sup> , <i>trp-6</i>	0.39	0.28	0.155	3.55
MI20	<i>Hyp</i> <sup>-</sup> , <i>trp-6</i>	—	0.26	0.166	3.45
MI21	<i>Hyp</i> <sup>-</sup> , <i>trp-6</i>	—	0.27	0.154	3.50
MI22	<i>Hyp</i> <sup>-</sup> , <i>trp-6</i>	0.39	0.28	0.152	0.7
MI24	<i>Hyp</i> <sup>-</sup> , <i>trp-6</i>	—	0.28	0.153	3.53
MI25	<i>Hyp</i> <sup>-</sup> , <i>trp-6</i>	0.41	0.288	—	3.54
MI26	<i>Hyp</i> <sup>-</sup> , <i>trp-6</i>	—	0.27	0.159	3.38
MI261	<i>Hyp</i> <sup>-</sup> , <i>trp-6</i>	—	0.28	0.159	3.32
MI27	<i>Hyp</i> <sup>-</sup> , <i>trp-6</i>	—	0.25	0.167	3.49
PAO677	<i>Hyp</i> <sup>-</sup> , <i>his-67</i>	—	0.273	0.156	3.36
PAO3031	<i>Hyp</i> <sup>-</sup> , <i>arg B18</i>	0.38	0.27	—	3.32
PAO3071	<i>Hyp</i> <sup>-</sup> , <i>argC54</i>	—	0.27	0.156	3.4
PAO3011	<i>Hyp</i> <sup>-</sup> , <i>leu-38</i>	—	—	0.154	3.45
PAO381211	<i>Hyp</i> <sup>-</sup> , <i>leu-38</i>	0.39	0.26	—	3.42

Mutants were grown in minimal medium containing 20 mM glycerol + 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> + 20 mM Hyp and the enzyme activities were determined as described in table 5. — No detectable enzyme activity.

Mutants MI25, PAO3031 and PAO381211 contained normal levels of epimerase, D-allo-Hyp oxidase and KGSA dehydrogenase but lacked PCHA deaminase activity. Therefore these mutants were classified as the deaminase<sup>-</sup> mutants.

When the cell extract of the mutant PAO3811 grown in a medium containing Hyp was examined for the presence of the enzymes of the pathway the epimerase and D-allo-Hyp oxidase activities were lacking. The low frequency of reversion to *Hyp*<sup>+</sup> phenotype (10<sup>-10</sup>) indicated that this strain is a deletion mutant for the genes responsible for the epimerase and D-allo-Hyp deaminase. The presence of fully induced levels of pyrroline hydroxy carboxylate deaminase and  $\alpha$ -ketoglutarate semialdehyde dehydrogenase in the cells extract of these mutant also supported this contention.

Mutant MI22 seemed to be blocked in  $\alpha$ -ketoglutarate semialdehyde dehydrogenase production. High levels of epimerase, D-allo-Hyp oxidase and deaminase could be detected in this mutant. The presence of a low level of 'non-specific'  $\alpha$ -ketoglutarate semialdehyde dehydrogenase in this mutant grown on DL-Ala, DL-Val, Pro and Glu suggested the absence of  $\alpha$ -ketoglutarate semialdehyde dehydrogenase specific to Hyp pathway.

Mutants unable to utilize Hyp as the sole source of carbon and nitrogen fell into 4 groups as defined by the absence of any one of the enzymes of Hyp pathway. It was possible to isolate mutants (2 numbers) that could grow with D-allo-Hyp as the sole

source of carbon and nitrogen. The growth rate of these mutants with D-allo-Hyp was similar to that with L-Hyp suggesting that these mutants permeated D-allo-Hyp like the cells of *P. putida* (Gryder and Adams, 1969) and *Pseudomonas* I (Jayaraman and Radhakrishnan, 1965a).

### Discussion

The biochemical studies on the Hyp degradative pathway in *P. aeruginosa* indicated that the synthesis of Hyp enzymes of pathway (figure 3) is inducible. The comparative examination on the regulation of the pathway enzymes in *P. aeruginosa*, *Pseudomonas* I and *P. putida* (Gryder and Adams, 1969) revealed that several features in the induction process are similar in these organisms. L- and D-allo isomers of Hyp were found to support the growth of *Pseudomonas* I (Kunthala and Radhakrishnan, 1964) and *P. putida* (Gryder and Adams, 1969). However, the results presented here indicate that there exists a barrier for the entry of D-allo-Hyp in *P. aeruginosa* and the permeability barrier can be overcome by treating the cells with EDTA. Similar observations were reported earlier by Wild *et al.* (1978) who showed that gram-negative enteric bacteria were not able to utilize D-amino acids. However these bacteria can utilize D-amino acids only after acquiring mutations responsible for the transport of the D-amino acids. Thus, these results indicate a key role for the transport systems in the dissimilation of imino acids.

Maximal levels of the enzymes of L-Hyp pathway was detected in the cells grown with Hyp. In the various mutants lacking the activity of epimerase, D-allo-Hyp oxidase, deaminase and KGSA dehydrogenase, L-Hyp nevertheless induced the remaining enzymes at levels comparable to the parental wild type. These results strengthen the argument that Hyp has an intrinsic inducing activity.

The extent of co-ordination of inducible enzymes of the pathway appears to show considerable variation in *Pseudomonas* and a generally emerging picture for a number of catabolic substrates is that of sequential induction of coordinate blocks of enzyme (Clarke and Ornston, 1975). The time of appearance of the enzymes of Hyp pathway failed to decide conclusively the manner in which the expression of the genes of the pathway is regulated. This could be due to the complication created by the occurrence of isoenzymes for D-allo-Hyp oxidase and  $\alpha$ -ketoglutarate semialdehyde dehydrogenase which could alter the time of enzyme appearance as well as levels of other enzymes in the pathway.

Among the classes of *Hyp*<sup>-</sup> mutants, it should be noted that epimerase<sup>-</sup> mutants were the most frequently occurring mutants. It was also possible to get many pyrrole hydroxy carboxylate deaminase<sup>-</sup> mutants. However, few D-allo-Hyp oxidase<sup>-</sup> and  $\alpha$ -ketoglutarate semialdehyde dehydrogenase<sup>-</sup> mutants were encountered. Since there exist multiple forms of D-allo-Hyp oxidase and  $\alpha$ -ketoglutarate semialdehyde dehydrogenase in this organism, it is not surprising that these classes of mutants are not easily detected in the screening procedure. The occurrence of redundancy in two of the four enzymes of the pathway made the genetic investigation exceedingly difficult. The occurrence of isoenzymes in *Pseudomonas* is not uncommon. The metabolic significance of the catalytically similar forms of enzymes is perhaps to augment the capacity with which the catabolic oxidation of organic compounds is accomplished efficiently in *Pseudomonas*.

Like many catabolic pathways in *Pseudomonas* (Kemp and Hegeman, 1968; Wheelis and Stanier, 1970; Leidigh and Wheelis, 1975) all the genes for the four

enzymes of the Hyp pathway are found to be co-transducible and form a small cluster in the chromosome. Surprisingly, the transductional studies showed that the gene for the separately inducible 'non-specific' D-allo-Hyp oxidase is part of the cluster. The phenomenon of superoperonic clustering of functionally related genes is not uncommon in *Pseudomonas aeruginosa* (Rosenberg and Hegeman, 1971) and in any event, the studies of catabolic pathways in *Pseudomonas* appear to show some unique features.

### Acknowledgements

The author wishes to thank Dr. Kunthala Jayaraman for valuable discussions, pertinent criticisms and suggestions. He is indebted to Dr. J. Jayaraman for offering a Certificate Course in Biological Chemistry Fellowship. He is also grateful to Dr. N. Clauson-Kass, Chemical Research Laboratory, Farum, Denmark for generously providing methyl, 2, 5-dimethoxy-tetrahydro-2-furorate used in the study.

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## Synthesis *in vitro* of cholesterol by mitochondria in the shrimp *Penaeus aztecus* (Ives)

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MS received 11 December 1979; revised 12 March 1980

**Abstract.** The incorporation of [<sup>14</sup>C]-acetate, [<sup>14</sup>C]-mevalonate and [<sup>14</sup>C]-desmosterol into cholesterol in the muscle mitochondria of the brown shrimp *Penaeus aztecus* (Ives) is more as compared to that in hepatopancreas. [<sup>14</sup>C]-Desmosterol is more efficiently incorporated into cholesterol in comparison with [<sup>14</sup>C]-acetate. The muscle mitochondria from males incorporated more [<sup>14</sup>C]-mevalonate into cholesterol than those from females, while the converse is true in the hepatopancreatic mitochondria.

**Keywords.** Cholesterol biosynthesis; muscle mitochondria; *Penaeus aztecus*.

### Introduction

Cholesterol biosynthesis is dependent on several endogenous and exogenous factors (Dempsey, 1974). Many dietary factors like linoleic acid, and  $\beta$ -sitosterol are known to retard or inhibit the synthesis in mammals (Goad, 1976). Earlier investigations (for review, see Goad, 1976) failed to demonstrate cholesterol synthesis in shrimp from simple precursors like acetate or mevalonate. In our laboratory, it was noticed that the brown shrimp *Penaeus aztecus* fed on algae-diet had reduced levels of cholesterol in tissues (Krishnamoorthy *et al.*, 1979). These observations prompted us to believe that earlier investigators had overlooked some interfering factors in the biosynthesis of cholesterol. It is felt that experiments on the particulate system may obviate these interferences. This paper presents the data on the synthesis of cholesterol *in vitro* by mitochondria, isolated from the brown shrimp tissues.

### Materials and methods

#### Substrates

(2,4-[<sup>14</sup>C]-)Desmosterol (30 Ci/mmol), (2-[<sup>14</sup>C]-)DL-mevalonic acid (10 Ci/mmol) and 1-[<sup>14</sup>C]-sodium acetate (15 Ci/mmol) were purchased from New England Nuclear, Boston, Massachussets, USA. All other chemicals were obtained from Sigma Chemicals, St. Louis, Missouri, USA.

#### Experimental animals

Adult brown shrimp (90-95 mm length) *P. aztecus* (Ives) were obtained from Davis Bayou in Ocean Springs, Mississippi and maintained in tanks with running sea water

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at 25°C. They were fed *ad lib* pelleted diet formulated in this laboratory (Gunter and Venkataramiah, 1975). This diet had 1.157 mg cholesterol per g dry wt. After maintaining the animals under these feeding conditions for 15 days, they were starved for one week before they were killed for analyses.

#### *Preparation of mitochondria*

All operations were performed at 0-4°C. The tail muscles and hepatopancreas (the so-called liver) of 15-20 shrimp were excised. Ten g of each tissue was rinsed with the homogenizing solution (0.25M sucrose, 6 mM EDTA, 20 mM imidazole-HCl buffer, pH 6.8), briefly blotted and homogenized in 20 volumes of a solution containing 0.1% (w/v) sodium deoxycholate (Hendler *et al.*, 1972) in a tissue grinder using 18 complete strokes at 600 g. The homogenate was filtered through a single thickness of cheese cloth and centrifuged at 600 g for 20 min in a Beckman J<sub>2</sub>-centrifuge. The sediment was discarded and the supernatant was then centrifuged at 18000 g for 45 min. After removal of the supernatant by aspiration, the mitochondrial pellet was resuspended in 20 ml of homogenizing medium, centrifuged at 18000 g and the pellet was collected. Finally an uniform suspension of the pellet in 5 ml phosphate (20 mM, pH 6.8) buffer was made. The protein content of the mitochondrial suspension was estimated colorimetrically (Lowry *et al.*, 1951) using human serum albumin (500 µg/ml) as the standard.

The purity of the mitochondrial preparations obtained was tested assaying the marker enzyme activities like succinic dehydrogenase (EC 1.3.99.1), cytochrome-c-oxidase (EC 1.9.3.1) and ouabain-sensitive Na<sup>+</sup>, K<sup>+</sup>-ATPase (EC 3.6.1.3) activity. Succinate dehydrogenase activity was determined spectrophotometrically with phenazine methosulphate and dichlororophenol indophenol as the electron acceptors as described by Arrigoni and Singer (1962). The activity was expressed as m units per mg protein; one unit being the amount of enzyme required to reduce 1 µ mol of the dye per min per mg protein at 25°C. Specific activity of cytochrome c oxidase (EC 1.9.3.1) was determined manometrically by measuring the oxygen uptake in the presence of added cytochrome c and a reagent to reduce the cytochrome c as it was oxidized (Slater, 1949) at 25°C. The activity was expressed as µl of oxygen consumed by 1 mg protein in 1 min. Ouabain-sensitive Na<sup>+</sup>, K<sup>+</sup>-ATPase activity was assayed according to Towle *et al.* (1976).

#### *Incubations with labelled precursors*

An incubation mixture containing cofactors: 60 µ mol glutathione, 17 µ mol glucose-6-phosphate, 25 µ mol MgCl<sub>2</sub>.6H<sub>2</sub>O, 23 µ mol NAD<sup>+</sup>, 7 µ mol NADH, 3 µ mol NADPH, 13 µ mol ATP, 200 µ mol nicotinamide and 100 µ mol glucose was prepared according to Teshima and Kanzawa (1976). This mixture (1.5 ml) in a test tube was mixed with 0.5 µ Ci of solvent-free precursor suspended in 1.5 ml of phosphate buffer containing 0.1 mg streptomycin and 0.1 mg penicillin G.

Incubation was started by the addition of 0.5 ml mitochondrial suspension containing 1 to 1.5 mg protein equilibrated at 28°C in a thermostat. After 3 h, 10 ml of 1:1 acetone-alcohol mixture was added to stop the reaction and the sterols were isolated by digitonin method (Idler and Baumann, 1952). The digitonides were collected by centrifugation and washed with ether. Finally, they were dissolved in dimethyl sulphoxide, and the free sterols were extracted with 1 ml hexane.

### Separation of sterols and autoradiography

The sterols in hexane ( $420 \mu l$ ) was subjected to thin layer chromatography on silica gel with the developer containing petroleum ether: ethyl ether and acetic acid 87.5:12.5:1) (Guerry, 1973). Cholesterol was run as a standard along with the unknowns. The zones were visualized in iodine vapour, scrapped and transferred into counting vials. Radioactivity in the gel scrapped from the cholesterol zones of the thin layer plate was determined after dissolving the sterol in  $150 \mu l$  absolute ethanol in 15 ml liqueflour and counted in a Packard Model 3375 scintillation spectrometer. The efficiency of counting was about 90%. Autoradiography was carried out by covering the thin layer chromatograms with x-ray film for 21 days.

### Results

The results of table 1 shows that the present homogenization procedure for isolating mitochondria reduced the apparent mitochondrial contamination in the sarcoplasmic

**Table 1.** Specific activities of marker enzymes in the fractions isolated from the tissues of the shrimp, *Penaeus aztecus*.

Enzyme	No. of observations	Units <sup>a</sup>	
		Cytoplasmic or sarcoplasmic fraction	Mitochondrial fraction
<i>Muscle</i>			
Succinate dehydrogenase activity <sup>b</sup>	8	$0.7 \pm 0.1$ (1.1)	$64 \pm 2.9$ (100)
Cytochrome-c-oxidase <sup>c</sup> activity	16	$0.6 \pm 0.09$ (1.2)	$49.8 \pm 2.1$ (100)
<i>Hepatopancreas</i>			
Succinate dehydrogenase activity <sup>b</sup>	8	$1.8 \pm 0.3$ (3.0)	$61 \pm 2.1$ (100)
Cytochrome-c-oxidase <sup>c</sup>	8	$1.86 \pm 0.39$ (4.0)	$46.9 \pm 2.5$ (100)

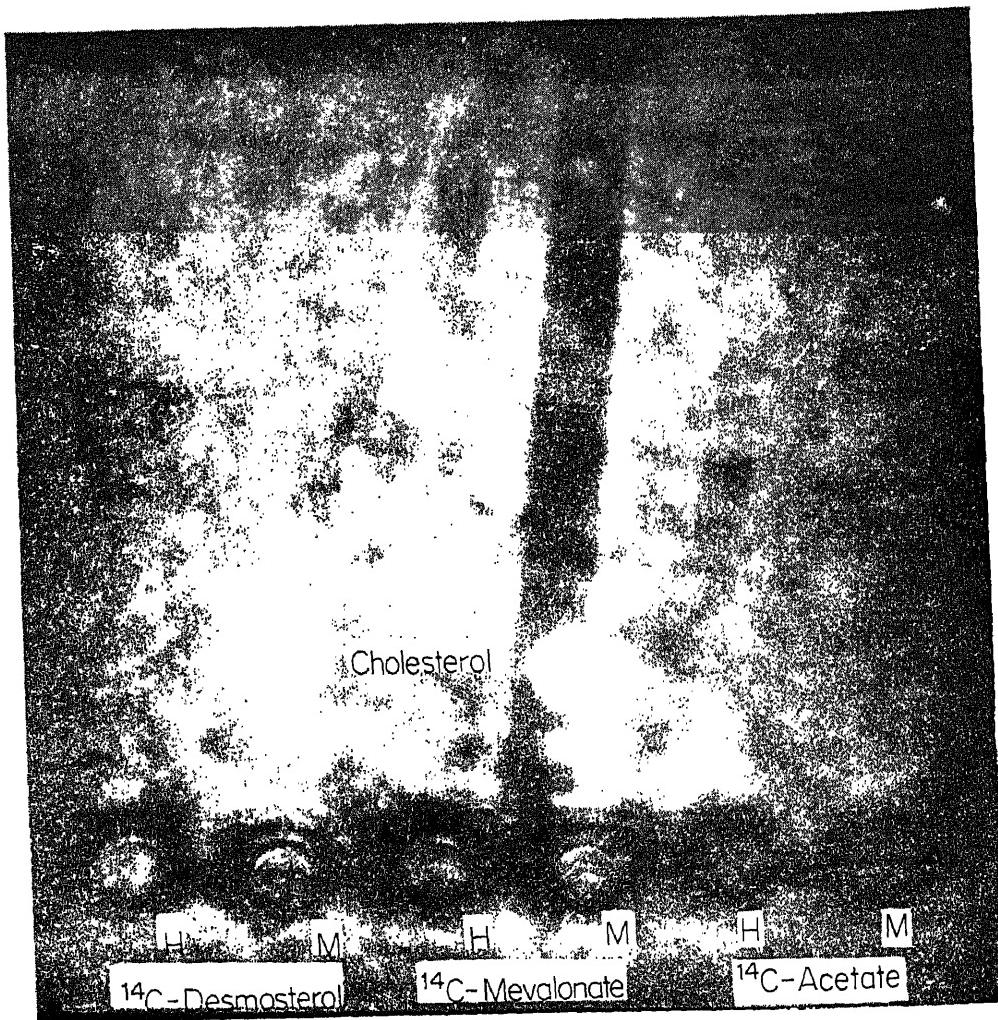
<sup>a</sup> mean  $\pm$  s.d.      <sup>b</sup> m units (see text for details).

<sup>c</sup>  $\mu l$  of  $O_2$  consumed per mg protein per min.

Number in parenthesis denote per cent purity.

(i.e., supernatant) fraction. Another possible contamination in muscle mitochondria is the proteins due to disruption of sarcolemma (Azzone, 1963). This was tested by the activity of the sarcolemmar marker enzyme, the ouabain-sensitive ( $Na^+$ ,  $K^+$ )-ATPase. In the mitochondrial fraction (see table 1), the Ouabain-sensitive ATPase activity was very low ( $< 0.12 \mu mol P_i/mg$  protein/min at  $25^\circ C$ ); hence the sarcolemmar contamination is minimum in the isolation process of the muscle mitochondria.

After the incubation of mitochondria, prepared from the muscle of hepatopancreas with the radioactive precursors, the incorporation of the radioactivity into unsaponifiable fraction of cholesterol was investigated. Figure 1 shows that significant radio-



**Figure 1.** Autoradiogram of the thin layer chromatogram. The mitochondria were incubated with [<sup>14</sup>C]-desmosterol, [<sup>14</sup>C]-mevalonate and [<sup>14</sup>C]-acetate and the reaction mixture was subjected to thin layer chromatography. H represents the mixture with the mitochondria of hepatopancreas and M is that of tail muscles of the shrimp.

activity was distributed in the cholesterol fraction obtained with muscle mitochondria. Only, the precursors mevalonate and desmosterol showed significant incorporation into cholesterol by the muscle mitochondria. Mitochondria of the hepatopancreas did not show significant radioactivity. The incorporation of acetate into cholesterol was poor in mitochondria from both the tissues.

The amount of labelled precursor incorporated into cholesterol was analyzed by counting the radioactivity of the cholesterol isolated by thin layer chromatography.

**Table 2.** Incorporation of labelled precursors into cholesterol by mitochondria of brown shrimp, *Penaeus aztecus*.

Mitochondria	Radioactivity incorporated <sup>a</sup> (%)		
	[ <sup>14</sup> C]-acetate	[ <sup>14</sup> C]-mevalonate	[ <sup>14</sup> C]-desmosterol
<i>Male</i>			
Muscle	2.03±0.18	40.60±11.63	56.31±6.36
Hepatopancreas	2.16±0.21	8.68± 0.93	18.67±2.64
<i>Female</i>			
Muscle	1.95±0.13	28.42± 1.51	46.39±4.78
Hepatopancreas	2.01±0.19	12.39± 2.36	15.86±2.23

<sup>a</sup> mean ± s.d., n = 6.

See Materials and methods for assay details.

The results are presented in table 2. Percentage incorporation of radioactive acetate into cholesterol was very low compared to other precursors. Desmosterol incorporation was higher than that of mevalonate, and the muscle mitochondria were more efficient than the mitochondria of hepatopancreas in incorporating these precursors into cholesterol.

The mitochondria from male shrimp showed greater rates than those of the female ( $P < 0.05$ ).

## Discussion

The ability of Crustaceans to synthesize sterols has been investigated by several authors, and hitherto it was established that they are incapable of *de novo* biosynthesis. It has been demonstrated that [ $1-^{14}\text{C}$ ]-acetate was not incorporated either into squalene or sterols by a crayfish, *Astacus astacus* (Zandee, 1962, 1964, 1966), a lobster *Homarus vulgaris* (Zandee, 1964) and a prawn, *P. japonicus* (Teshima and Kanazawa, 1971a). It was also demonstrated that a brine shrimp *Artemia salina*, a prawn *P. japonicus*; a lobster *Panulirus japonica* and a crab *Portunus tritaberculatus* failed to utilize labelled mevalonate for sterol elaboration (Teshima and Kanazawa, 1971 a, b). It was therefore believed that shrimp rely upon a dietary source of sterol or dealkylate dietary phytosterols into cholesterol (Teshima and Kanazawa, 1976; Teshima, 1972). Furthermore, in most of the earlier studies (Zandee, 1966; Teshima and Kanazawa, 1971a; Teshima, 1972), [ $^{14}\text{C}$ ]-acetate or mevalonate was either injected into the blood stream or incubated with tissue slices. In general when [ $^{14}\text{C}$ ]-acetate was injected or incubated, it was poorly incorporated into the sterols, and in a few cases it was apparently not incorporated at all into sterols, though some fatty acids were appreciably labelled showing that the animals were able to utilize the [ $^{14}\text{C}$ ]-acetate. The mevalonate was also not incorporated in many cases and hence, the authors deduced that cholesterol synthesis is absent in shrimp. In the light of these observations the present results demonstrating the occurrence of cholesterol synthesis *in vitro* by the shrimp mitochondria are interesting.

The poor incorporation of [<sup>14</sup>C]-acetate into sterols as observed here is now rationalized on the basis of recent work on the role of  $\beta$ -hydroxy- $\beta$ -methyl glutaryl CoA-reductase (EC 1.1.1.34) in the control of mammalian sterol synthesis (Brown *et al.*, 1973). Clearly, if  $\beta$ -hydroxy- $\beta$ -methyl glutaryl-CoA reductase is also the rate-limiting step in sterol synthesis in shrimp, then the incorporation of [<sup>14</sup>C]-acetate will depend upon the activity of this enzyme.

Starvation markedly reduces the activity of  $\beta$ -hydroxy  $\beta$ -methyl glutaryl CoA reductase (Ramasarma, 1974). Probably, for this reason, low incorporation rates of [<sup>14</sup>C]-acetate, into sterols were obtained in the present study. When mavalonate (a product of the  $\beta$ -hydroxy  $\beta$ -methyl glutaryl-CoA reductase) was incubated with mitochondria, the incorporation was not subjected to this regulation (Richard and Rodwell, 1974; Dempsey, 1974). Desmosterol, an intermediate in the *de novo* synthesis of cholesterol was converted into cholesterol. The specific conversion rates were more with desmosterol than with mevalonate. These results are not surprising as the conversions of desmosterol into cholesterol have been demonstrated in the prawn *P. japonicus* (Teshima and Kanazawa, 1971b).

$\beta$ -Hydroxy  $\beta$ -methyl glutaryl-CoA reductase activity is controlled by a number of dietary factors (Brown *et al.*, 1973; Goad, 1976). The activity may be suppressed by dietary cholesterol or  $\beta$ -sitosterol. Apparently, studies with mitochondria reduce the influence of such factors and therefore when incubated with mitochondria, mevalonate was easily incorporated. The results thus demonstrate that mitochondria of shrimp *P. aztecus* are capable of synthesising cholesterol.

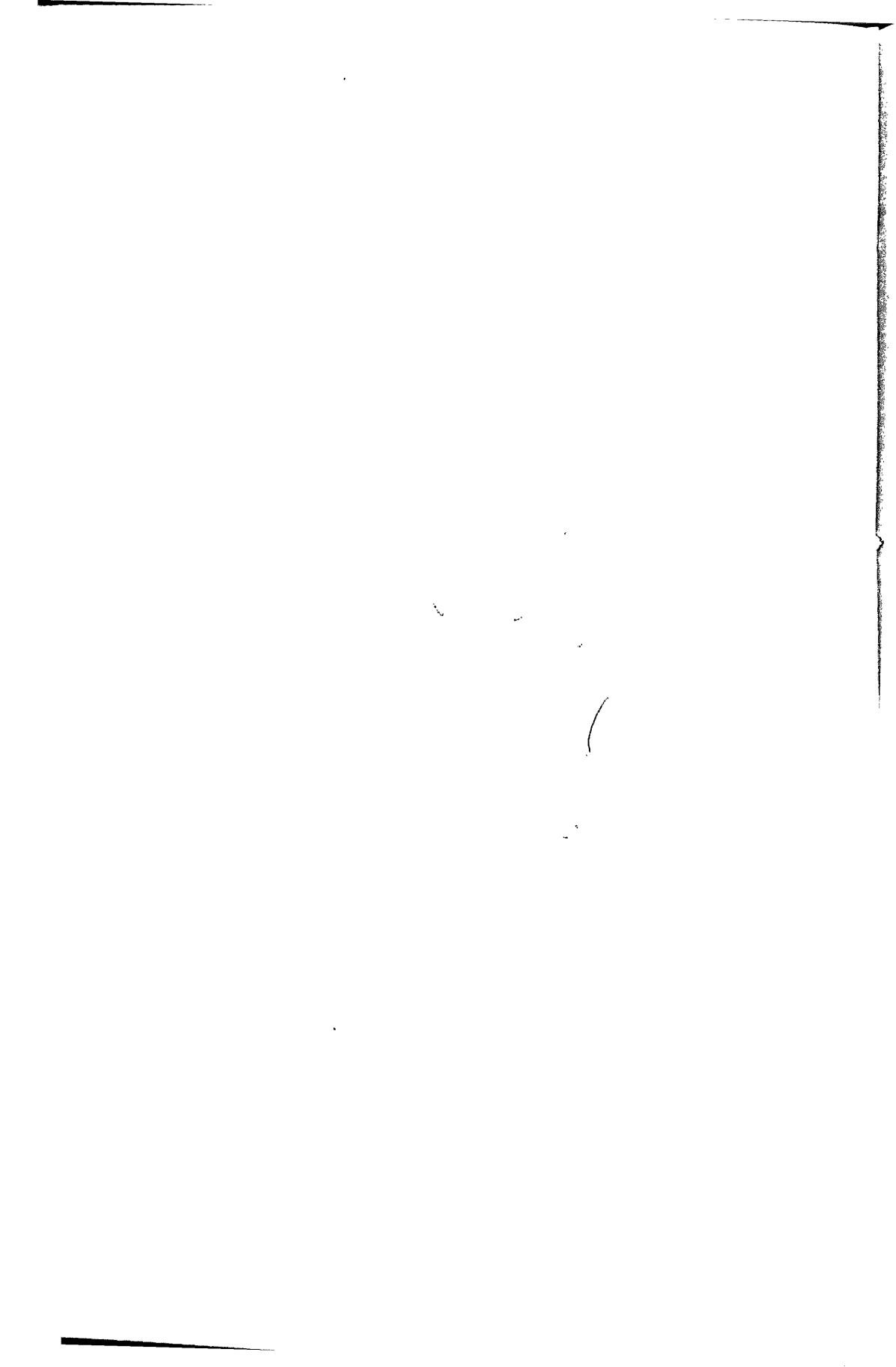
### Acknowledgements

The authors thank Mr John D. Valleau for his help in isotope experiments and Miss Patricia Biesiot for maintaining the animals.

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## ***In vitro* hemolytic activity of *Plasmodium berghei* on red blood cells**

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MS received 31 December 1979; revised 9 May 1980

**Abstract.** A suspension of *Plasmodium berghei* obtained by lysis with saponin of red blood cells from an infected rat showed high hemolytic activity, when incubated *in vitro* with normal rat red blood cells. The hemolysis was a temperature-dependent process and was dependent on the concentration of the parasite. Plasma of *Plasmodium berghei* infected albino rats also possessed lytic activity.

**Keywords.** *Plasmodium berghei*; hemolytic factor; anemia.

### **Introduction**

Severe anemia is a major symptom of malarial infection (reviewed by Krier and Leste, 1967 and Roth, 1978). Although lysis of infected erythrocytes during the release of merozoites is believed to be one of the causes for anemia, many workers have demonstrated that loss of erythrocytes exceeded the percentage of the infected cells. Other mechanisms suggested for the large decrease of erythrocytes include concomitant infection (Ludford *et al.*, 1969) alterations of the erythropoietic mechanisms (Frankenburg and Greenblatt, 1977), autoimmunity (Soni and Cox, 1974, 1975; Lustig *et al.*, 1977) reticuloendothelial hyperphagocytosis directed against host erythrocytes (Zuckerman *et al.*, 1973; Roth and Herman, 1979), alterations in the erythrocyte membrane and hemolysis by the parasite products (Fife *et al.*, 1972; Seed *et al.*, 1976; Holz *et al.*, 1977; Klein *et al.*, 1977).

The present communication summarizes the results of a preliminary investigation on the hemolytic activity of *P. berghei* and plasma from the infected albino rats.

### **Materials and methods**

#### *Animals*

Eight weeks old male albino rats (Charles Foster strain, 35-40 g), were used in the present study. The animals were housed in plastic cages and received food and water *ad lib.* throughout the experiment.

#### *Parasite preparation*

The animals were infected intraperitoneally with  $1 \times 10^6$  parasitized RBC. At peak parasitaemia, blood was collected in acid citrate dextrose by cardiac plexus puncture and the plasma was stored at  $4^\circ\text{C}$ . The parasites were harvested from the infected

erythrocytes by the saponin lysis methods of Zuckerman *et al.* (1967). The parasitic material was thoroughly washed and suspended in 0.15 M saline to a protein content of 2.0 mg/ml. Erythrocyte forms of *P. berghei* were maintained by weekly transfusion of infected blood into normal animals.

#### *Normal rat erythrocyte suspension*

Erythrocytes were collected from normal rat, washed thrice with 0.15 M saline, suspended in the same medium and adjusted to a final concentration of  $25 \times 10^7$  cells/ml. Hemolysate obtained by 1:4 dilution of this suspension with distilled water had an absorbance of  $0.70 \pm 0.01$  at 540 nm.

#### *Assay of hemolytic activity*

Aliquot samples of the parasite preparations were mixed with 0.5 ml of normal erythrocyte suspension in small tubes and the final volume was made to 2.0 ml with 0.15 M saline. The mixture was incubated at 37°C for 30 min, and then centrifuged at 2,000 g for 10 min. The absorbance of the supernatant was measured at 540 nm against a reagent blank in which the parasite was substituted by saline. The percentage of hemolysis was calculated by normalizing against the absorbance of a mixture of 0.5 ml red blood cells and 1.5 ml distilled water, which showed complete hemolysis. For the assay of the hemolytic activity in infected plasma, aliquots of infected plasma were used instead of the parasite.

#### *Separation of hemolytic activity by gel filtration*

A suspension of the parasite was sonicated in an MSE Ultrasonic disintegrator for 30 min at 1.2 amps output at 4°C. Two ml of the sonicate containing 6.0 mg protein/ml was loaded on to a Sephadex G-200 column (2.5×40.5 cm) previously equilibrated with 0.15 M saline. The elution was performed with saline and 2.0 ml samples were collected. Protein was monitored in the effluents at 280 nm and the fractions were assayed for the hemolytic activity.

#### *Infected plasma*

Aliquots of plasma (20 mg protein) from infected rats were loaded in a Sephadex G-200 column (2.5×40 c.m.) and the fractions were collected as described above.

### **Results**

#### *Hemolysis of rat red blood cells by *P. berghei* suspension and infected plasma*

Figure 1a shows the data on hemolysis of normal rat red blood cells with different aliquots of the parasite suspension. The rate of hemolysis was relatively low when the aliquots of the parasite suspension in the range were 0.05-0.20 ml. Thereafter the hemolysis increased at a rapid rate reaching a limiting value.

Rate of hemolysis with the infected plasma was relatively faster at lower concentrations (figure 1b). With 1.0 ml of the plasma, about 20% hemolysis was observed.

#### *Hemolytic activity as a function of incubation temperature*

Figure 2 shows the hemolytic activity of *P. berghei* suspension incubated with normal rat red blood cells at 25, 30 and 37°C. The maximum activity was observed

at 37°C. The hemolytic activity was slightly lesser at 30°C but a significant decrease in activity was observed at 25°C.

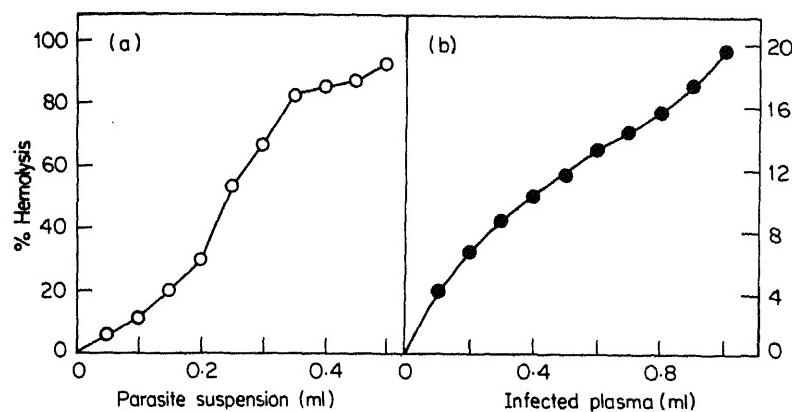


Figure 1. (a) Hemolytic activity of whole parasite on normal rat RBC. (b) Hemolytic activity of infected rat plasma on rat RBC.

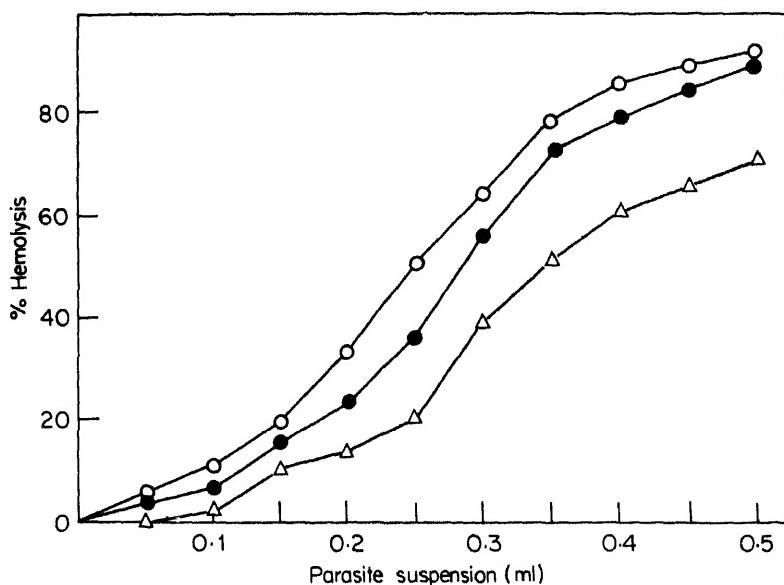


Figure 2. Effect of temperature on hemolytic activity of whole parasite 25°C,  $\Delta$ ; 30°C, ●; 37°C, ○;

*Hemolytic activities of sephadex fractions of the sonicated parasite and infected plasma*

The proteins of the sonicated parasite were separated into two peaks (figure 3). The hemolytic activity was recorded in the first major peak. Proteins of the infected plasma were resolved into 3 peaks. The lytic activity was confined to the middle peak which contained bulk of proteins (figure 4).

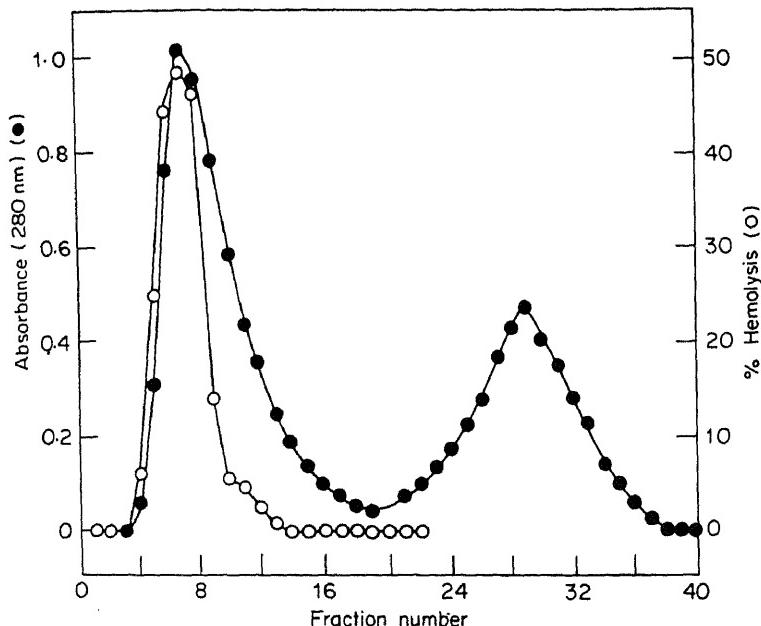


Figure 3. Sephadex G-200 gel filtration pattern of sonicated *P. berghei* and the hemolytic activity of eluted fractions, absorbance at 280 nm, ●, absorbance at 540 nm, ○.

*Effect of the lytic factor on sheep red blood cells*

To find out whether the hemolytic activity of *P. berghei* is confined to rat red blood cells, the parasite suspension was incubated with sheep erythrocytes. The latter were, however, found to be equally susceptible to the lytic action.

**Discussion**

The results presented herewith clearly demonstrate the presence of a hemolytic factor(s) in *P. berghei*, which can lyse normal rat and sheep erythrocytes. The lytic factor could be extracted with aqueous medium and hemolytic action was a temperature dependent process. These observations substantiate the early reports of Fife *et al.* (1972), on the presence of a similar factor in *P. knowlesi*. The hemolytic factor of *P. berghei*, however, appears to be different from the *P. knowlesi* factor in its molecular weight. The *P. berghei* lytic factor was eluted just after the void volume in the first protein peak and has a relatively higher molecular weight ( $>150,000$ ) than

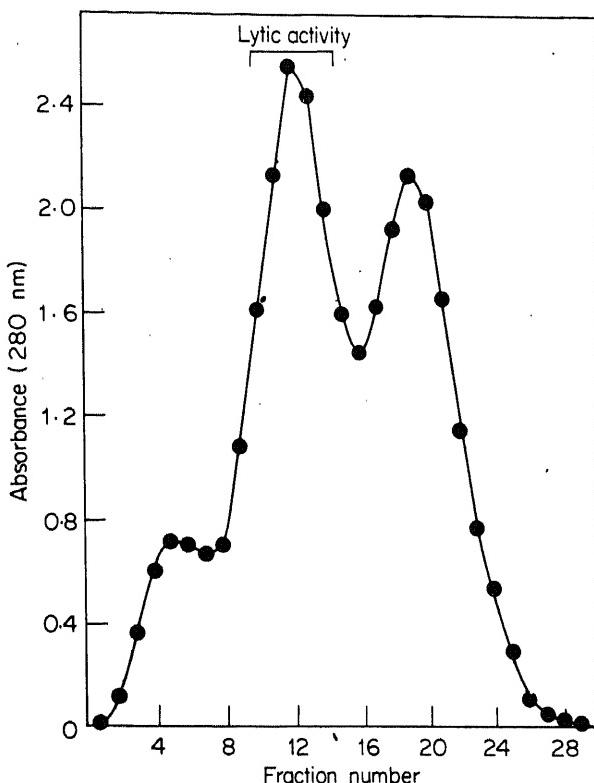


Figure 4. Sephadex G-200 gel filtration pattern of *P. berghei* infected rat plasma and the hemolytic activity of eluted fractions.

that of the *P. knowlesi* lytic factor, which is around 500 only. It is, however, likely that in the case of *P. berghei*, the active constituent is bound to a macromolecule, possibly a protein.

The *P. berghei* lytic factor differs from the *P. knowlesi* lytic factor also in the pattern of hemolysis kinetics. Whereas with the *P. berghei* lytic factor, the hemolytic activity starts immediately after the reaction mixture is kept for incubation, with *P. knowlesi* lytic factor, there is a lag phase of about 4 h before hemolysis starts.

The observations on the presence of a lytic factor in *P. berghei* infected rat plasma is, again, in accordance with the report of Fife *et al.* (1972) on the presence of a similar factor in *P. knowlesi* infected monkey plasma. The plasma lytic factor from the two sources appeared to have similar molecular size as both were recovered from the sephadex columns in association with the 7S proteins. The second peak containing lytic activity corresponds to 7S proteins. However, it may be pointed out that the activity of lytic factor of the infected monkey plasma could not be demonstrated unless the plasma was resolved by gel filtration where as the activity of infected albino rat plasma could be shown even in the unresolved condition. likely to play a role in the development and pathogenesis of anemia encountered with malarial infection.

### Acknowledgements

The authors are grateful to Dr Nitya Nand, Director, and Dr. A. B. Sen, Division of Parasitology, for their keen interest in the work.

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## **Effect of sciatectomy and induced ammonia stress on $Mg^{2+}$ -adenosine triphosphatase activity in frog tissues**

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MS received 7 January 1980; revised 19 March 1980

**Abstract.**  $Mg^{2+}$  dependent—adenosine triphosphatase activity has been studied in the muscle, brain, kidney and liver tissues of frog, *Rana hexadactyla* (Lesson) after sciatectomy and induced chronic ammonia stress. The enzyme activity decreased in the tissues of the denervated frog. The activity of the enzyme increased in all the tissues of the normal and denervated frogs except in the denervated muscle when ammonium lactate was infused intraperitoneally.

**Keywords.** Sciatectomy;  $Mg^{2+}$ -adenosine triphosphatase; ammonia stress; *Rana hexadactyla*.

### **Introduction**

The energy required by the tissues for maintenance of normal trophic state is obtained by metabolic activity. If the trophic influence of the nerve is impaired, a disturbance in energy metabolism occurs, either because the energy cannot be obtained by the normal procedures from the usual sources or its utilization for trophic functions is impaired (Bass, 1962). It has been reported that there is an elevation in ammonia content in gastrocnemius muscle after sciatectomy in frog (Krishnamoorthy *et al.*, 1971; Sreeramulu Chetty, 1978). Ammonia is known to interfere in energy metabolism (Chow and Pond, 1972). In order to ascertain whether the impairment in energy metabolism is merely due to a lack of trophic influence of the nervous system or due to an increased ammonia build up, a comparative study on the effects of denervation and ammonia toxicity on  $Mg^{2+}$ -adenosine triphosphatase (EC 3.6.1.3) was undertaken.

### **Materials and methods**

Batches of frogs were unilaterally denervated under aseptic conditions, as described elsewhere (Muralikrishna Das and Swami, 1968). The muscle with intact innervation was designated as the contralateral muscle, while the neurectomized muscle was designated as the denervated muscle and the muscle of the normal frog was designated as normal muscle. Three doses of ammonium lactate were administered intraperitoneally in 0.5 ml physiological saline and the doses were designated as

Mild dose— 1.5 mg of ammonia/kg body weight

Moderate dose— 2.5 mg of ammonia/kg body weight

Sublethal dose— 5.0 mg of ammonia/kg body weight.

Ammonium lactate was administered to the normal as well as denervated frogs once a day for one week while the control animals received 0.5 ml of physiological

saline. The liver, kidney, brain and muscle tissues were isolated, homogenized in cold 0.25 M sucrose and centrifuged in a Remi centrifuge at 1400 g for 10 min to remove cell debris. The supernatant was used as the source of the enzyme.  $Mg^{2+}$ -adenosine triphosphatase was estimated by the method of Tirri *et al.* (1973) with slight modifications and the liberated inorganic phosphate was estimated by the method of Fiske and Subbarow (1925). Protein measurement was carried out by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

### Results and discussion

On sciaticectomy, a decrease in the  $Mg^{2+}$ -ATPase activity levels was observed in the liver, kidney, brain, contralateral and denervated muscles (table 1). About 30%

**Table 1.** Activity levels of  $Mg^{2+}$ -ATPase in the different tissues of normal and denervated frogs.

Tissue	Normal	Contralateral	Denervated
Muscle	3.6±0.5	2.9±0.2 <sup>a</sup>	2.5±0.7 <sup>b</sup>
Brain	9.8±0.5	—	8.9±0.8 <sup>c</sup>
Liver	7.5±0.5	—	5.2±0.4 <sup>d</sup>
Kidney	11.5±1.0	—	8.2±0.3 <sup>d</sup>

(Values are expressed in  $\mu$  mol of  $P_i$  formed/mg protein/h).

Each value is  $\pm SD$  of 6 observations.

$a = p < 0.01$      $b = p < 0.05$      $c = p < 0.02$      $d = p < 0.001$

decrease in activity was seen in liver, kidney and denervated muscle of denervated frogs as compared to the corresponding tissues of control frogs; while a slight decrease in ATPase activity was observed in the brain and contralateral muscle of the denervated animals. On administration of ammonium lactate, the normal and contralateral muscles have shown an increase in  $Mg^{2+}$ -ATPase activity at all three dosages was observed; whereas, the activity in the denervated muscle was unchanged (table 2).

An increase in the  $Mg^{2+}$ -ATPase activity levels was observed in the liver and kidney tissues of both the normal and denervated frogs (table 2). The magnitude of increase was larger in the tissues of denervated frog than in the normal frog. While McCaman (1961) reported decreased activity of  $Ca^{2+}$  and  $Mg^{2+}$  dependent ATPase in skeletal muscle following denervation. Dobrinina *et al.* (1972) observed an increase in  $Mg^{2+}$ -ATPase activity. The present study has shown a decrease in enzyme activity in the denervated muscle consistent with McCaman's results. Michelazzzi *et al.* (1957) also observed a similar decrease during denervation atrophy in guinea pig indicating a decrease in the energy release by this enzyme. It is reported that the diminished activity of glycolytic pathway, and the decrease in the activity of tricarboxylic acid cycle enzymes due to the disintegration of mitochondria in the denervated muscle, resulted in decreased ATP production (Swami and Satyanarayana, 1976). Hence, it can be inferred that the decrease in  $Mg^{2+}$ -ATPase activity observed

**Table 2.** Effect of exogenous ammonia on levels of Mg<sup>2+</sup> ATPase in different tissues and in denervated frogs.

Tissue	Dose of ammonium lactate			
	None (control)	Mild	Moderate	Sublethal
<i>Muscle</i>				
Normal	3.6±0.5	4.5±0.5 <sup>a</sup>	5.2±0.5 <sup>b</sup>	5.4±0.5 <sup>b</sup>
Contra-lateral	2.9±0.2	5.2±0.7 <sup>b</sup>	6.0±0.8 <sup>b</sup>	5.8±0.4 <sup>b</sup>
Denervated	2.5±0.7	2.5±0.7 <sup>e</sup>	2.5±0.7 <sup>e</sup>	2.8±0.6 <sup>e</sup>
<i>Brain</i>				
Normal	9.8±0.5	11.9±1.3 <sup>b</sup>	14.1±2.2 <sup>e</sup>	16.8±0.9 <sup>e</sup>
Denervated	8.9±0.8	11.2±1.1 <sup>e</sup>	15.9±1.8 <sup>b</sup>	16.8±0.6 <sup>b</sup>
<i>Liver</i>				
Normal	7.5±0.5	8.3±0.6 <sup>a</sup>	11.8±1.2 <sup>b</sup>	13.2±1.1 <sup>b</sup>
Denervated	5.2±0.4	6.2±0.7 <sup>c</sup>	9.5±0.7 <sup>b</sup>	12.5±0.8 <sup>b</sup>
<i>Kidney</i>				
Normal	11.5±1.0	11.7±0.8 <sup>e</sup>	14.9±1.1 <sup>b</sup>	15.8±0.4 <sup>b</sup>
Denervated	8.2±0.03	9.0±0.8 <sup>a</sup>	10.9±1.1 <sup>a</sup>	12.1±2.0 <sup>b</sup>

(Values are expressed in  $\mu$  mol of P<sub>i</sub> formed/mg protein/h).Each value is  $\pm$ SD of 6 observations.<sup>a</sup> = p < 0.01    <sup>b</sup> = p < 0.001    <sup>c</sup> = p < 0.02    <sup>d</sup> = p < 0.05    <sup>e</sup> = Not significant.

in the denervated muscle may be due to the impairment in the synthesis of ATP generating system.

On the administration of ammonium lactate, ATPase activity increased in the tissues of both the normal and denervated frogs (except in the denervated muscle) indicating a general stimulatory effect of ammonia. Hawkins *et al.* (1973) have reported similar increase of ATPase activity in the brain and liver tissues when rats were exposed to ammonia toxicity. Schenker *et al.* (1967) also have suggested that ammonia has a stimulatory effect on ATPase activity resulting in the decreased concentrations of ATP in the brain of ammonia-intoxicated rats. The present study indicates a contrasting pattern of influence of the denervation and ammonia stress on ATPase activity in the gastrocnemius muscle, suggesting that the energy metabolism of this tissue is impaired. It has been reported that sciatectomy results in elevation of ammonia production (endogenous) in the muscle (Krishnamoorthy *et al.*, 1971; Sreeramulu Chetty, 1978). This endogenous elevation could not stimulate ATPase activity in the muscle. Similar failure in producing an increase in ATPase activity by exogenous ammonia in muscle in contrast to other tissues is observed suggesting the importance of neutral influence for its action in the muscle.

#### Acknowledgement

One of the authors (S.R.C.) is thankful to Council of Scientific and Industrial Research, New Delhi, for the award of a fellowship.

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## Effects of dynamic impacts on human bones

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**Abstract.** Flat bones of human skeleton were subjected to dynamic indentation with ball indenters. The impacted surface was studied under high magnification and also by using the technique of multiple beam interferometry. The impulse caused the pile up of material at a little distance from the edge of the indent. The diameter of indent is found to increase as fourth root of the energy of impact. Bone structure also has the tendency to minimize the damage caused by external forces. There was about 90% recovery in deformation in the depth of indents due to internal stresses created inside the bone by the impact.

**Keywords.** Bone; impacts; surface distortion; recovery; Hertz theory.

### Introduction

An important function of the bones is to provide protection to underlying tissues against different types of impulsive forces. Considerable work has been reported on the study of the fracture, cracks, and strain pattern of bones by subjecting a cadaver to impulsive forces with large and heavy weights (Glasser, 1960). Some work has also been done on microscopic deformation of bones due to impacts of small light bodies (Bonfield and Li, 1966). Interferometric technique has been used to study the distortion of human bone surface due to diamond pyramidal vicker indentation (Ramrakhiani *et al.* 1979). The present paper describes interferometric studies of microscopic deformation of human bones due to dynamic indentation with small spherical balls, and the recovery of the deformed portion of the bones.

### Methods

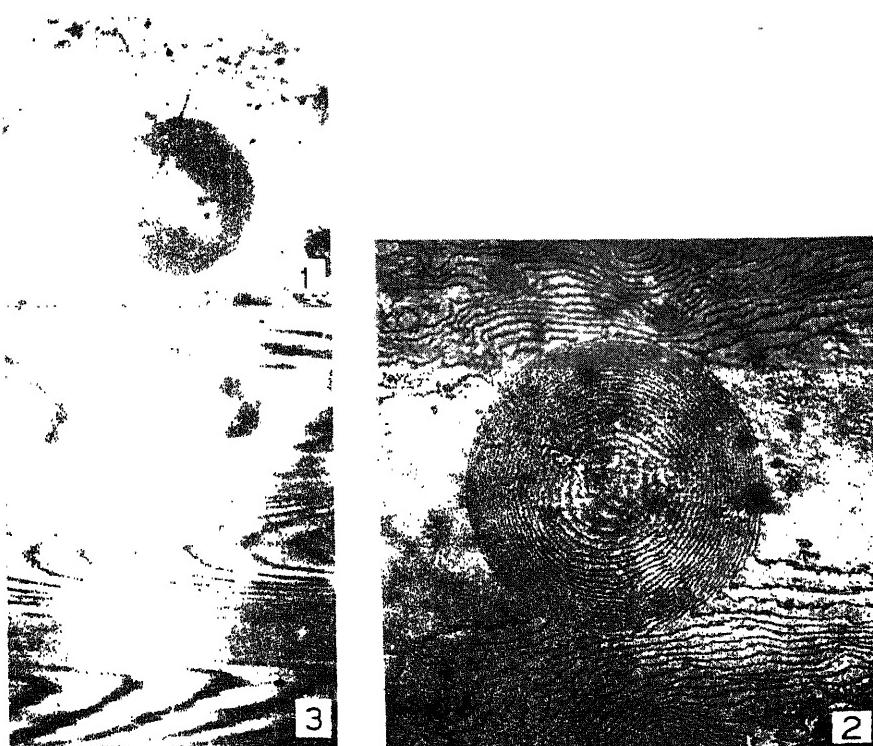
The bone specimens were cleaned and polished and cut to suitable sizes which consist of compact layer of bone of approximate size 1 cm × 1 cm. Their outer surfaces were coated with a thin layer of silver by vacuum coating technique in 'Hindhivac' 12A4 vacuum coating unit. The specimens were placed in the chamber and then it was evacuated with the help of rotary and oil diffusion pumps. Then the silver placed in one of the filaments was evaporated by heating; it condenses on the specimen surfaces and gives a uniform coating. This increased their reflectivity, and the surfaces could then be examined easily under reflected light. These were

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Abbreviation used: FECO, fringes of equal chromatic order

then subjected to dynamic indentation with three different indenters of known mass. All the three indenters had a small ball fixed at their tips. These balls were made of hardened steel having a diameter of 0.8 mm ( $1/32''$ ). The indenter was allowed to fall on the specimen from a known height. To avoid the air current and limit the path of the indenter, it was dropped on the specimen through a glass tube.

Impacts were made with all the three indenters by allowing them to fall from different heights. Sufficient care was taken to minimize the vibration of the indentation unit so that the entire energy of impact was given to the specimen. The indentations were examined under high magnification in Meopta TAH 3 microscope and their diameters were measured with the help of the micrometer eye piece. The minute distortion of the bone surfaces due to the impacts was studied by the multiple-beam interferometric technique (Tolansky, 1960). The interference fringes were obtained by matching the specimen with a semisilvered optically plain glass plate and placing them in a jig under a microscope. The wedge angle between the matching flat and the silvered bone surface was adjusted with the help of the jig. Fizeau fringes were obtained by using monochromatic light and the fringes of equal chromatic order (FECO) were obtained by projecting this fringe pattern on the slit of a constant deviation spectrograph and replacing monochromatic light by white light.



**Figure 1-3.** 1. Dynamic ball indent on human bone ( $m=50$ ). 2. Fizeau fringes on ball indent ( $m=150$ ). 3. FECO along diameter of the indent.

## Results

The impact on the surface of a bone with a spherical ball resulted in a spherical depression observable under the microscope (figure 1). The size of the impressions was found to increase with the velocity of impact as well as with the mass of the indenter.

The indentation with very low energy (2500 erg or less) was not perceptible. The smallest indent discerned was due to the energy of impact of 2680 erg. The impression was circular and of diameter 0.165 mm and of negligible depth. As the energy of impact was increased the indents became more and more prominent. Sufficiently deep impressions were obtained by high energy impacts. Rebound of the indenter was also observed in the case of impacts of high energy of the order of  $10^5$  erg or more.

### *Surface distortion studies*

The smallest indent observed was due to energy of 2680 erg. A little plastic deformation was produced which could be observed with some difficulty. Fizeau fringes were slightly deviated at the indent site but fringes of equal chromatic order clearly showed the depression of depth (2500 Å). Indents obtained with greater impact energies resulted in greater plastic deformation and were observed with ease under a microscope. Fizeau fringes on the indents were circular but not smooth (figure 2). This indicates that the surface features are not changed by indentation. Figure 3 details the FECO along a diameter of the indent. At the centre of the indent the fringes are narrow and curved towards the red showing a depression. Then there are also very narrow horizontal fringes which are not visible. This is due to large wedge angle. The fringes outside the pit appear to be curved towards the violet. It implies that the surrounding bone surface has been elevated. This piling up is of the order of 100 Å and the peak is at a little distance from the edge of the pit.

The piling up of the surrounding material due to dynamic indentation, at some distance, can be explained easily. As the indenter strikes the bone, some bone material is pressed away making space for the indenter. As the force is impulsive, some material is displaced away from the point of indentation with some velocity. However, further displacement of the bone material is resisted by the internal forces. This, consequently, leads to a pile up at a little distance from the edge of the indent.

### *(b) Recovery studies*

As a moving indenter strikes a bone, the bone is deformed and internal stresses are created in it. These resist further penetration of the indenter and push it back, and also try to bring the substances to its original form. This causes the deformation to recover. The recovery has been found in the depth of indents only and not in the diameter. The depth to which the bone has been penetrated can be calculated according to the following relation. The depth of penetration,

$$t = \frac{D - \sqrt{D^2 - d^2}}{2} \quad (1)$$

where  $d$  is the diameter of indent and  $D$  is the diameter of the ball indenter.

The actual depth of indent was measured by FECO pattern on the indent. The depth of indent,

$$t' = \frac{(n_1 - n_2) \lambda_1 \lambda_2}{(2\lambda_1 - \lambda_2)} \quad (2)$$

where  $\lambda_1$  and  $\lambda_2$  are two wave lengths selected on FECO pattern and  $n_1$  and  $n_2$  are number of fringes between them due to the different wedge thicknesses. (Tolansky, 1960)

The depth of penetration and depth of indent for different indents are given in table 1. It is seen that depth of indent is much smaller than depth of penetration.

**Table 1.** Dynamic ball indentation.

Weight of the indenter (g)	Height (cm)	Observed diameter of indent (mm)	Calculated diameter of indent (mm)	Depth of penetration (mm)	Depth of indent (mm)
0.638	10	Undetectable	0.19	—	—
0.638	20	0.249	0.218	0.02	0.00069
0.638	30	0.29	0.237	0.0276	0.00104
0.638	40	0.3	0.25	0.0296	0.00187
0.638	60	0.37	0.272	0.046	0.00585
0.638	80	0.41	0.288	0.0573	0.0049
0.638	100	0.44	0.3	0.0668	0.008677
1.365	1	Undetectable	0.087	—	—
1.365	2	0.165	0.16	0.00872	0.0002566
1.365	3	0.203	0.174	0.0133	0.0008224
1.365	4	0.213	0.184	0.01467	0.00073
1.365	10	0.276	0.22	0.025	0.003
1.365	20	0.29	0.25	0.0276	0.0029
1.365	30	0.379	0.276	0.048	0.0039
1.365	60	0.39	0.317	0.051	0.006
1.365	80	0.401	0.336	0.0546	0.0073
1.365	100	0.456	0.35	0.0723	0.00735
4.766	3	0.235	0.22	0.017	0.000354
4.766	5	0.3	0.247	0.0296	0.0008
4.766	10	0.33	0.284	0.036	0.00265
4.766	20	0.43	0.327	0.0635	0.0054
4.766	30	0.5	0.35	0.089	0.00756
4.766	50	0.53	0.39	0.1018	0.008
4.766	80	0.576	0.43	0.125	0.01855

Radius of ball indenter ( $r$ ) = 0.396 mm.

There is about 90% recovery and depth of indent is only 1/10th of the depth to which the indenter has penetrated. Because of the recovery in the depth, the radius of curvature of indent is also much greater than that of the indenter. It is found to be about 10 times i.e. 4 mm in comparison to 0.4 mm that of the indenter. This shows that if the momentary forces do not exceed the breaking strength of bone, very little

permanent damage is caused in comparison to the deformation at the moment of impulse.

#### *Comparison with Hertz theory*

The concept given by Hertz about the stress distribution set up by static indentation with a spherical ball on a solid, for elastic deformation, has been extended to the case of dynamic impact by considering the rate of change of momentum of the impacting bodies (Tasi and Kolsky, 1967). It gives the radius of contact circle during the indentation of a spherical ball on a plane surface in terms of elastic constants of the material and the indenter as

$$a = 0.987 \left[ W \left( \frac{1 - \sigma_1^2}{E_1} + \frac{1 - \sigma_2^2}{E_2} \right) \right]^{1/5} (VR)^{2/5} \quad (3)$$

where  $W$  is the weight of the indenter,  $V$  is the velocity of impact,  $R$  is radius of indenter,  $\sigma_1$  and  $\sigma_2$  are the Poisson ratio of substance and indenter, respectively, and  $E_1$  and  $E_2$  are the Young's moduli of substance and indenter, respectively.

This has been derived by considering the material as an ideal plastic material and deformation purely elastic. These conditions are not satisfied in the present case; even then, it is interesting to find the agreement of the observed diameters with the diameters given by the theory. The observed and calculated diameters are given in table 1. The variation of diameter with impact energy is shown in figure 4. It is seen

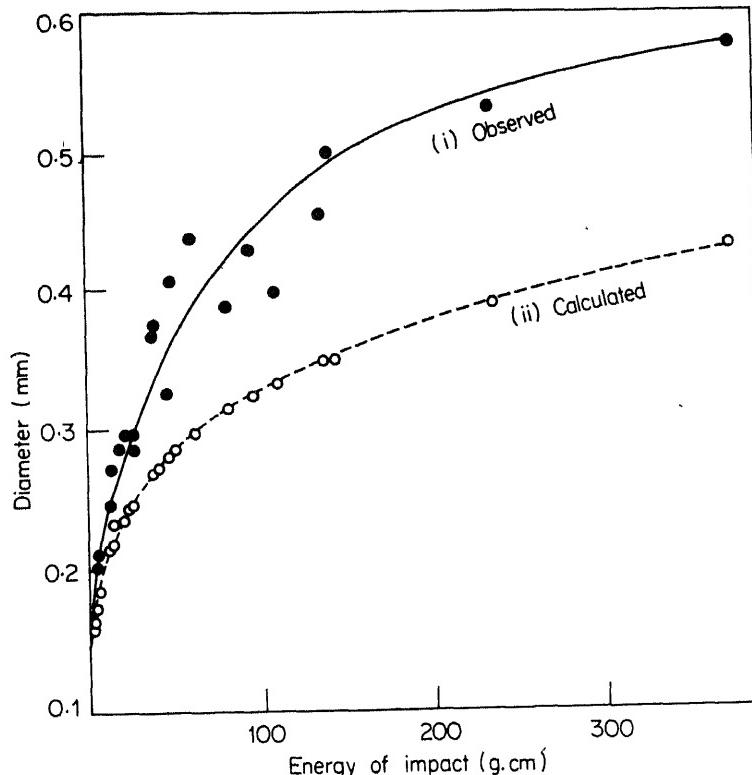


Figure 4. Energy of impact vs indent diameter curves.

that all the points for observed diameters are not falling on the mean curve, which may be due to the non-homogeneity of the bone material. The increase in diameter of the indent with increase in impact energy is obvious and is in agreement with the theory. However, the observed and calculated diameters are not exactly the same. Mostly, the observed diameter is greater than the calculated diameter, but diameter of the smallest indent, surprisingly, agrees with the calculated value and is perhaps due to the fact that the deformation is more or less elastic. Both the curves (i) and (ii) start from this point.

According to the theory, the diameter increases as the fifth root of the energy, as can be seen from eq. 3. The experimentally observed diameters increase approximately as the fourth root of the energy instead of the fifth root. This is accounted for by the plastic deformation of the material.

### Conclusion

The investigations have revealed that the impacts on bones with small bodies try to push the bone material away and in turn the bone material also tries to throw the impacting bodies back as a result of internal stresses created during the impact. Bone is capable of giving rise to large amounts of internal stresses which in turn lead to tremendous recovery of the deformed portion, and thus tries to reduce the damage by the applied forces.

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## **Proteins of the brain and body wall in larvae of *Drosophila melanogaster***

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MS received 11 February 1980

**Abstract.** Proteins of the brain and body wall cells of third instar larvae of *Drosophila melanogaster* have been examined by two-dimensional gel electrophoresis. Out of over 600 [<sup>35</sup>S]-labelled peptide spots seen in brain or body wall extracts, 517 were common to both; 61 spots were unique to brain and 66 unique to muscle. Glycoproteins were identified by soaking the gels in radioactive iodinated Concanavalin-A. Forty four Con-A binding glycoproteins were identifiable in the brain and 41 in the muscle extracts. Out of these, 8 glycoproteins of the brain and 8 of muscles appear to be tissue-specific.

**Keywords.** Proteins; glycoproteins of brain; muscle; *Drosophila* larvae; 2-dimensional gel electrophoresis.

### **Introduction**

Since the isolation by Benzer (1967) of non-phototactic mutants, a variety of behavioural mutants of *Drosophila melanogaster* have been found (Reviews by Benzer, 1971, 1973; Pak and Pinto, 1976; Ward, 1977). Identification of biochemical lesions in neurological mutants is expected to throw light on molecular mechanisms underlying behaviour. Of particular interest, from this point of view, are the proteins of the nervous system. Two-dimensional gel electrophoresis provides a convenient way of looking for altered proteins in mutants (O'Farrell, 1975). Our group is interested in temperature-sensitive paralysed mutants of *Drosophila* some of which appear to be affected in nerves or muscles (Siddiqi and Benzer, 1976; Ikeda *et al.*, 1976; Wu *et al.*, 1978). As a ground work for comparing mutants with normal flies, we have examined the two dimensional (2D) maps of proteins and Con-A binding glycoproteins of the brain and body wall of wild type larvae.

Tissiers *et al.* (1974) and Arking (1978) analyzed the proteins of *Drosophila* larvae by one-dimensional electrophoresis and Rodgers and Shearn (1977) have examined the proteins of imaginal discs by two-dimensional electrophoresis. Using short periods of labelling, Tissiers *et al.* (1974) and Arking (1978) found that the protein patterns changed markedly at different stages of development. As our primary purpose was to maximise the chances of detecting mutational alterations, we fed the

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Abbreviations used: SDS; Sodium dodecyl sulphate, PMSF: Phenyl methyl sulphonyl fluoride, NP40: Non ionic detergent (P40), Con.A: Concanavalin A; 2 D, two-dimensional; DNase; deoxyribonuclease; RNase, ribonuclease.

larvae on radioactive sulphur for prolonged periods to ensure uniform labelling. In this paper we present an account of [ $^{35}\text{S}$ ]-labelled proteins and Con-A binding glycoproteins from larval brain and body wall. The body wall preparation consists predominantly of larval muscles.

### Materials and methods

#### Strain

The experiments were carried out with the wild type strain Canton Special (CS) of *Drosophila melanogaster*.

#### Materials

#### Chemicals and solutions

**Chemicals:**  $\text{Na}_2^{35}\text{SO}_4$  and  $\text{Na}^{125}\text{I}$  were obtained from Bhabha Atomic Research Centre, Trombay. Ampholines were purchased from LK Broma, Sweden, ultrapure urea from Schwarz Mann, New York USA, Nonionic detergent (NP40) from Fluka ulm, W. Germany and  $\beta$ -mercaptoethanol from E. Merck, Darmstadt, W. Germany. Sigma Chemical Co., St. Louis, MO, USA, supplied Tris-base, deoxyribonuclease (DNAase), ribonuclease (RNAase), agarose, Concanavalin-A (lyophilized powder) and cytochrome-c. Sodium dodecyl sulphate (SDS) was obtained from Pierce, Illinois, USA; Acrylamide, N-N'-methyl bisacrylamide and N,N,N',N' tetramethylene diamine, from Eastman Kodak, Rochester, New York, USA and glycine from Fischer Scientific Co., Philadelphia, USA. The rest of the chemicals were of analaR quality obtained from Sarabhai Merck, Baroda.

**Solutions:** The following working solutions were essentially as described by O'Farrell (1975). (1) Lysis buffer:— 9.5 M urea, 2% (w/v) nonidet P40, 2% ampholines, pH 3.5-10 and 5%  $\beta$ -mercaptoethanol (b) Sonication buffer: 0.01 M Tris-HCl pH 7.4, 5 mM  $\text{MgCl}_2$ , 50 ug/ml pancreatic RNAase (c) DNAase-solution: 1 mg/ml solution in 0.01 M Tris-HCl pH 7.4 and 1 mM  $\text{MgCl}_2$ . (d) Phenyl methyl sulphonyl fluoride (PMSF):— 40 mM stock in 95% ethanol (3) sample overlay solution:— 9M urea, 1% ampholines pH 3.5-10. (f) Acrylamide solution for the 1st dimension — 28.38% w/v acrylamide and 1.62% bisacrylamide (g) Acrylamide solution for the 2nd dimension (29.2% acrylamide and 0.8% bisacrylamide). *Drosophila* ringer (Ikeda and Kaplan, 1970):  $\text{NaCl}$  128 mM,  $\text{KCl}$  4.7 mM,  $\text{CaCl}_2$  1.8 mM,  $\text{Na}_2\text{HPO}_4$  0.74 mM,  $\text{KH}_2\text{PO}_4$  0.35 mM.

#### Radioactive labelling of larvae

Larval proteins were labelled uniformly with [ $^{35}\text{S}$ ] by feeding the larvae with yeast grown on  $^{35}\text{SO}_4$ . The feeding medium (0.2 ml) containing 10% dextrose, 5% sucrose and 1.5% agar was dispensed in 5 cm  $\times$  0.7 cm glass tubes. Fifty microlitres of radioactive yeast ( $3 \times 10^8$  dpm) was added and the medium was melted to allow mixing with yeast. Fifty early second instar larvae (about 60 h of age) identified by size and anterior spiracles (Bodenstein, 1965), were placed in each tube and kept at 23-24°C in a moist chamber. After 88 h of feeding, the larvae developed into late 3rd instar. The incorporation of [ $^{35}\text{S}$ ] under these conditions was about  $6 \times 10^6$  dpm per larva. Increasing radioactivity beyond 0.23 m C/ $100 \mu\text{l}$  of medium causes visible damage to larvae. They remain small and stunted and die within 2 days.

### *Preparation of sample for electrophoresis*

The labelled larvae were washed in *Drosophila* ringer and fed on cold feeding medium containing unlabelled yeast for 2 h in order to chase the undigested radioactive yeast. Each larva was again washed and transferred to a drop of ringer on a cavity slide kept on ice. The larvae were dissected under a microscope. The brains and body walls were cleansed of all extraneous tissues and transferred to separate 4 cm  $\times$  0.6 cm glass microhomogenizers.

Body walls of 10 larvae were homogenized in 60  $\mu$  l homogenising buffer (sonication buffer 25  $\mu$ l, DNAase 25  $\mu$ l, PMSF 10  $\mu$ l). 48 mg of urea and 90  $\mu$ l of lysis buffer were then added to make the final urea concentration 9M. About 50 brains were homogenized in 45  $\mu$ l homogenizing buffer (sonication buffer, 20  $\mu$ l; DNAase 20  $\mu$ l, PMSF 5  $\mu$ l) followed by addition of 38 mg urea and 75  $\mu$ l of lysis buffer.

The usual incorporation of [ $^{35}$ S] in the tissues was about  $6 \times 10^5$  dpm/body wall and about  $6 \times 10^4$  dpm/brain. The extracts were either loaded on the gel immediately or stored at  $-40^\circ\text{C}$  for subsequent use. These extracts could be stored upto a week without appreciable deterioration.

### *Electrophoresis*

Two dimensional polyacrylamide gel electrophoresis was carried out essentially as described by O'Farrell (1975) with certain modifications which are noted below:—

A 11.5 cm isofocussing gel containing Ampholines in the pH range 3.5-10 in 4% acrylamide was made in a 13  $\times$  0.25 cm (internal diameter) glass tube. The gel was overlaid with lysis buffer and pre-run at 200 V for 20 min followed by 300 V for 25 min and 400 V for 35 min. The upper cathode compartment of the electrophoresis apparatus contained 0.02 M degassed NaOH and the lower compartment contained 0.01 M  $\text{H}_3\text{PO}_4$ .

About 30  $\mu$ l of body wall extract or 70  $\mu$ l of brain extract containing  $8 \times 10^5$  dpm and about 25  $\mu$ g protein, was loaded on the gel and overlaid with 20  $\mu$ l of sample overlay solution. The proteins were electrofocussed at 400 volts for 16 h followed by 800 V for 1 h at room temperature ( $23^\circ\text{C}$ ).

The cylindrical gel was equilibrated in sodium dodecyl sulphate-buffer on a reciprocal shaker for 90 min at room temperature and laid on a 14  $\times$  11  $\times$  2.25 cm SDS slab gel with a linear gradient of 8.5% to 14% polyacrylamide, and a 2.5 cm top layer of 5% acrylamide stacking gel. Five ml of 1% agarose in sodium dodecyl sulphate buffer was poured over the cylindrical gel and allowed to solidify. Electrophoresis in the second dimension was carried out towards the anode in a vertical gel apparatus at 100 V for 6.5 h, using 0.025 M Tris-HCl in 0.192 M glycine and 0.1% sodium dodecyl sulphate at pH 8.3 as the electrode solution.

The gels were fixed overnight in 50% methanol and 10% acetic acid. The fixed gels were expanded to original size in 7% acetic acid for 4 h, and dried under evacuation. Dried gels were radiographed on Kodak Medical X-ray film in dry chambers. The usual exposure time for a gel loaded with  $8 \times 10^5$  dpm was three weeks.

### *Labelling glycoproteins with iodinated Concanavalin-A*

Concanavalin-A was labelled with [ $^{125}\text{I}$ ], using chloramine-T as described by Hunter and Greenwood (1962). Iodinated Con-A was purified on a 8  $\times$  0.9 cm column of

Sephadex G-75 by washing with 0.2 N acetic acid, neutralized with 1 M Tris-HCl buffer (pH 8) and stored at -40°C. The specific activity of labelled Con-A was 0.1 m Ci/mg.

Fixed gels of brain or body wall extracts (unlabelled) were washed with distilled water giving three changes for 1 h each time and equilibrated with buffer containing 0.1 M Tris HCl pH 7, 1 mM MnCl<sub>2</sub> and 1 mM CaCl<sub>2</sub>. Each gel was soaked in 100 ml of fresh buffer containing  $4 \times 10^7$  dpm of [<sup>125</sup>I]labelled Con-A and 1 mg/ml of cytochrome c for 24 h (Burridge, 1976). After repeated washing with buffer these gels were dried and radiographed.

#### <sup>35</sup>S-labelled yeast

*Saccharomyces cerevisiae* was labelled according to the procedure of Graham and Stanley (1972). Ten mCi of Na<sub>2</sub><sup>35</sup>SO<sub>4</sub> was added to a 20 ml culture of growing yeast in a sulphur-free medium at a cell density of  $3 \times 10^6$ /ml. After 18 h the labelled cells were centrifuged, washed thrice with sterile saline, and resuspended in 3.5 ml saline. The level of radioactivity in the suspension was about  $6 \times 10^9$  dpm/ml.

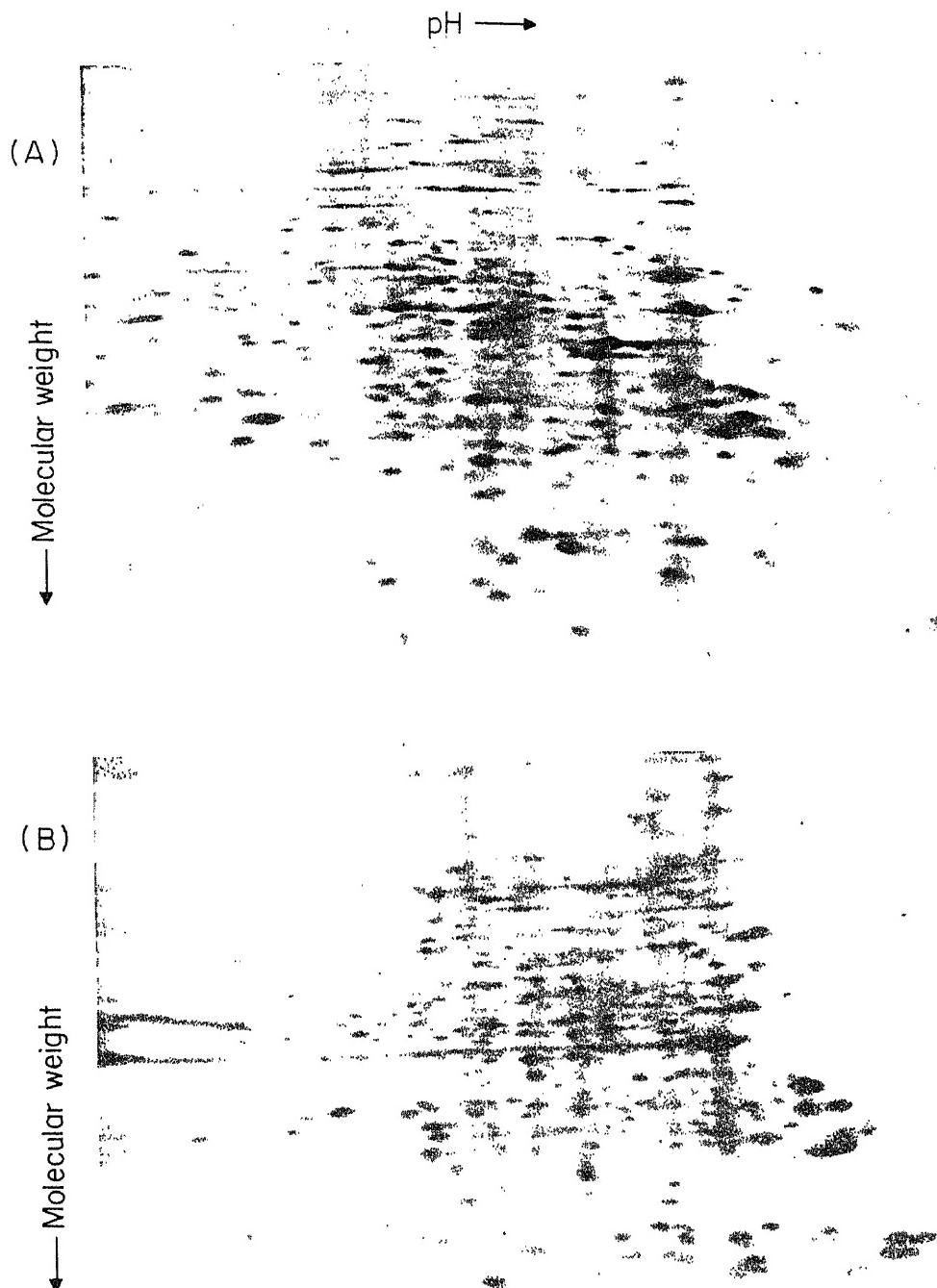
### Results

The two dimensional gel patterns of peptides from brain and body wall of 3rd instar larvae of *Drosophila* are shown in Figure 1. The larvae were labelled for 88 hours and extracts were made from 10 body walls or 50-60 brains. One might, therefore, expect that differences arising from stage of development (Tissiers *et al.*, 1974; Arking, 1978) would be greatly reduced. There is, nevertheless, a certain amount of variability in these gels. A part of this variation undoubtedly arises from the fact that the detection of faint spots near the threshold of visibility greatly depends upon the extent of exposure. In addition, there is some inescapable variation in the technique of electrophoresis itself (O Farrell, 1975; Rodgers and Shearn, 1977). In order to make a comparison of wild type and mutant patterns more reliable, we have attempted an objective assessment of the reproducibility of total protein patterns by comparing independent gels to construct idealized maps for peptides of body wall and brain.

#### Comparison of brain and body wall gels

The autoradiographs were enlarged to 10" × 12" prints. Using prominent peptides as landmarks, the entire gel was subdivided into six segments and the spots were arbitrarily numbered. One gel, each of brain and body wall from three independent experiments were then carefully compared for the presence or absence of each numbered spot and the results were tabulated in serial order. In spite of some variations in size and intensity of spots in different gels, the 'positional identity' of a spot could be determined with the help of surrounding landmarks. Thus, spots having the same 'relative position' in the different gels of brain and body wall, carried the same numbers. The overall reproducibility of spots in the brain and body wall gels may be gauged from table 1.

The body wall profile was then compared with the brain profile and the spots were classified into three groups: a) spots common to brain and body wall, b) spots unique to brain and c) spots unique to body wall. Spots that had the same 'relative positions' in at least two out of three gels of both brain and body wall, were called 'common spots'. These are shown in figure 2. Two additional gels of brain and body



**Figure 1.** Two dimensional gels of [<sup>35</sup>S]-labelled proteins of *Drosophila melanogaster* larvae: A, proteins of brain. B, proteins of body wall. Molecular weights and pH were estimated in parallel runs. These are to be taken as approximate. Approximately  $8 \times 10^5$  dpm of [<sup>35</sup>S]-counts equivalent to about 25  $\mu$ g. protein was loaded on each gel. The gel was exposed to X-ray film for 3 weeks.

**Table 1.** The reproducibility of peptide spots in brain and body wall two dimensional gels.

	Brain	Body wall
Spots present in all 3 gels	520	514
Spots present in 2 gels out of three	91	105
Total	611	619
Spots present in 1 gel out of three	39	27

Only spots present in at least two gels out of three, are considered reproducible. Spots present in just one gel are considered irreproducible, and are not included in the total.

wall were examined for unique spots. We have considered a spot unique to either brain or body wall, if it was seen in at least three out of five gels of one kind and none of the other. The distribution of brain specific and body wall specific spots is shown in figure 3. The differences between these tissues in selected segments of the gel are presented in figure 4. Out of the total of about 620 spots recorded, 66 were unique to body wall and 61 unique to brain. About 80% of the spots were common to both tissues.

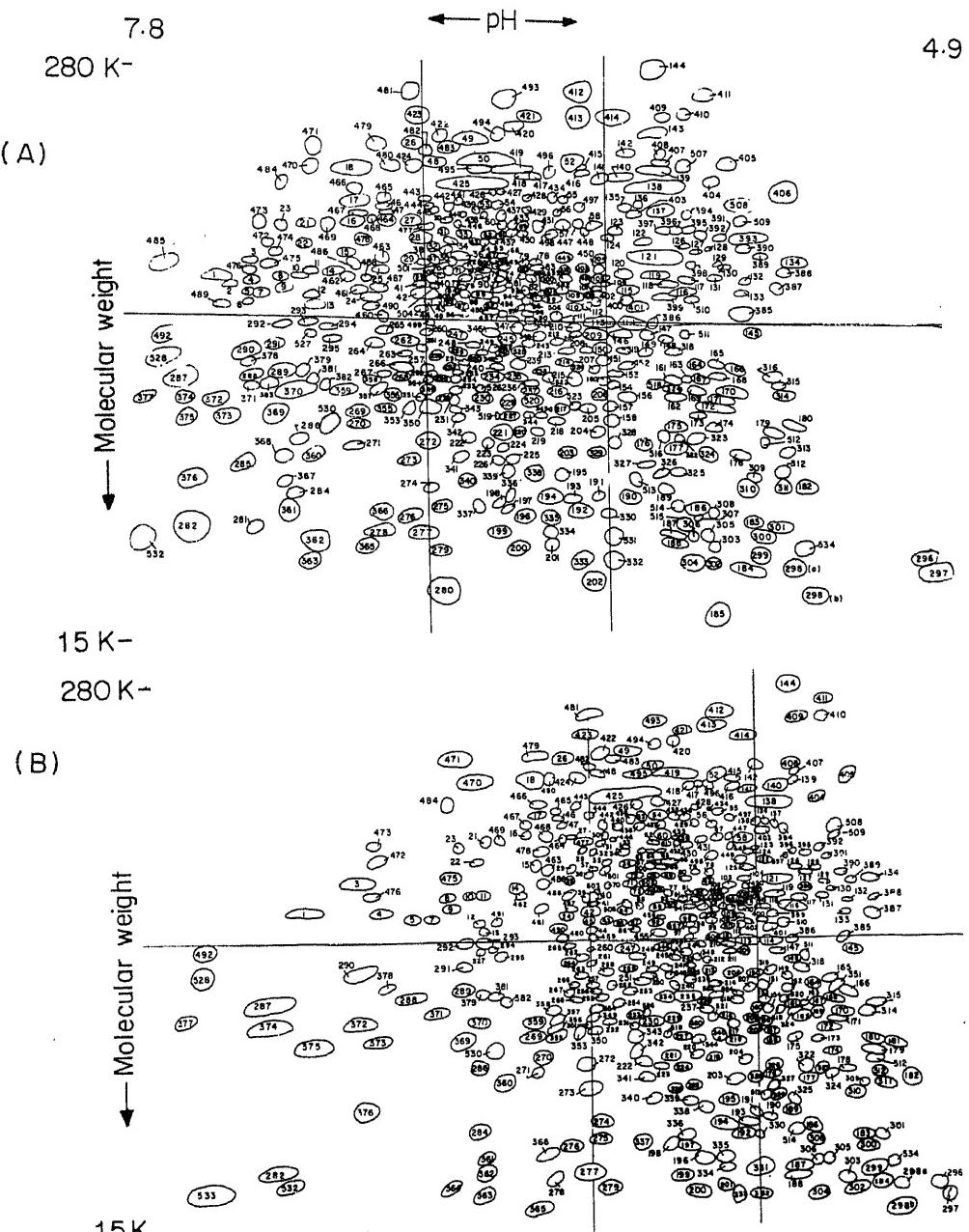
**Table 2.** Reproducibility of glycoprotein spots in two dimensional gels.

	Brain	Body wall
Spots present in all 3 gels	30	16
Spots present in 2 gels out of 3	14	25
Total	44	41
Spots present in 1 gel out of 3	6	8

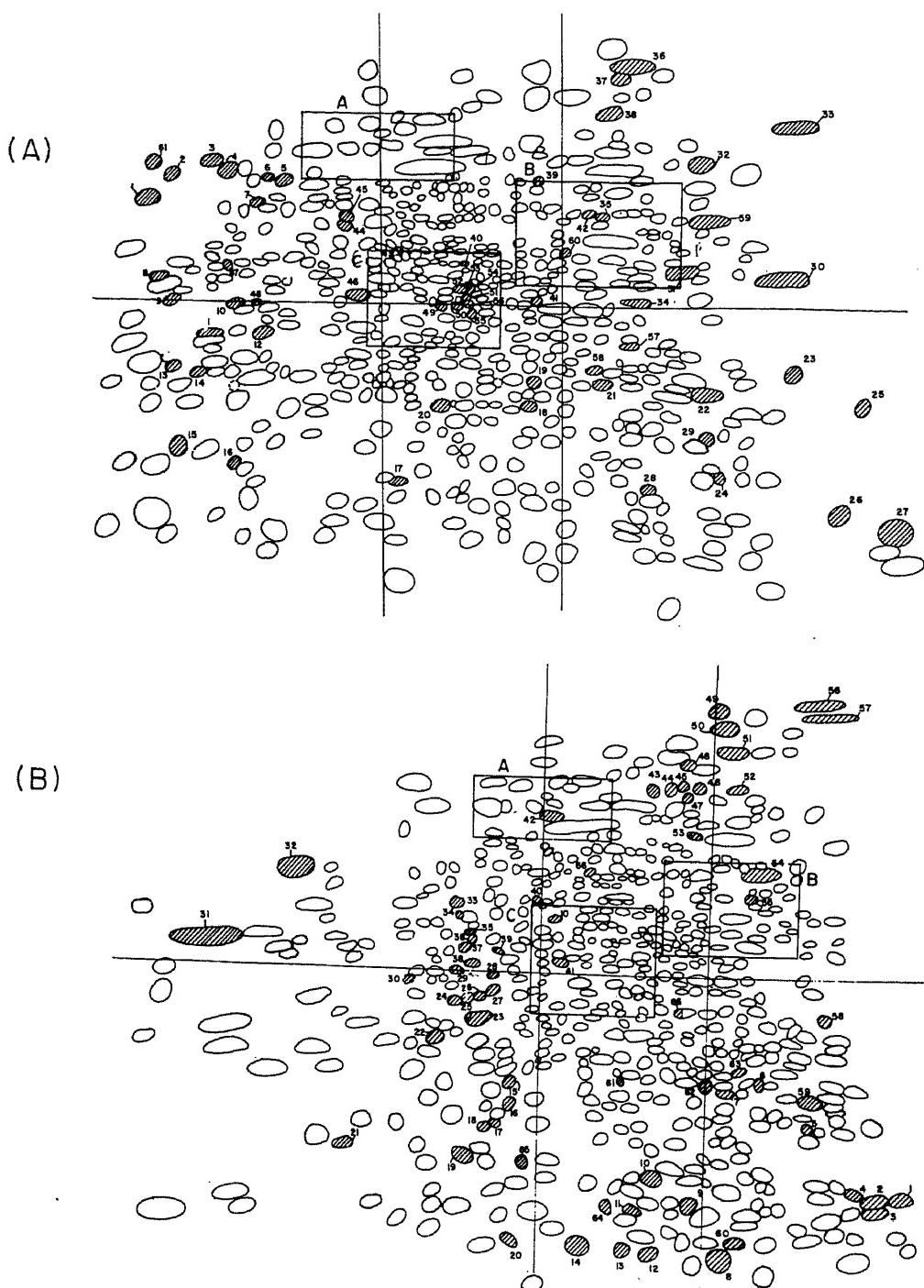
Spots present in just 1 gel are not included in the total as they are considered irreproducible.

### Glycoproteins

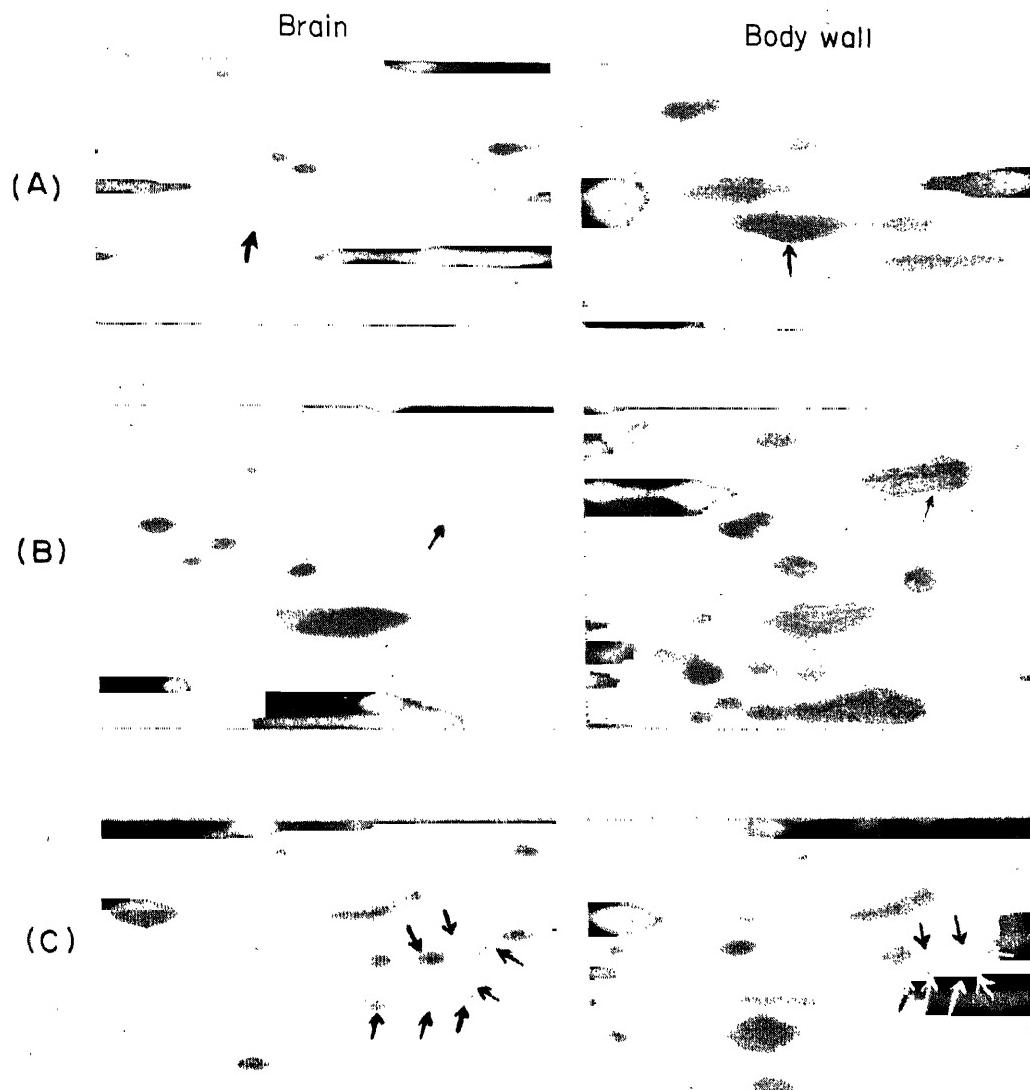
Concanavalin-A binding glycoproteins were visualized by soaking two dimensional gels of extracts of brain and body wall of unlabelled larvae in con-A labelled  $^{125}\text{I}$ . Glycoprotein spots were compared in the same manner as total proteins. Two gels each of body wall and brain were examined and spots present in two out of three gels were considered reproducible. These spots were classified as common and unique. Common spots were present in at least 2 out of 3 gels of both brain and body wall. Unique spots were present in at least 2 out of three gels of one kind and none of the other. The reproducibility of glycoprotein spots is presented in Table 2 and the distribution of common and unique spots is shown in Figure 5. The body wall extracts had 41 spots of which 8 were unique. In brain, 8 out of 44 spots were unique. Twenty seven spots appeared to be common to both the tissues.



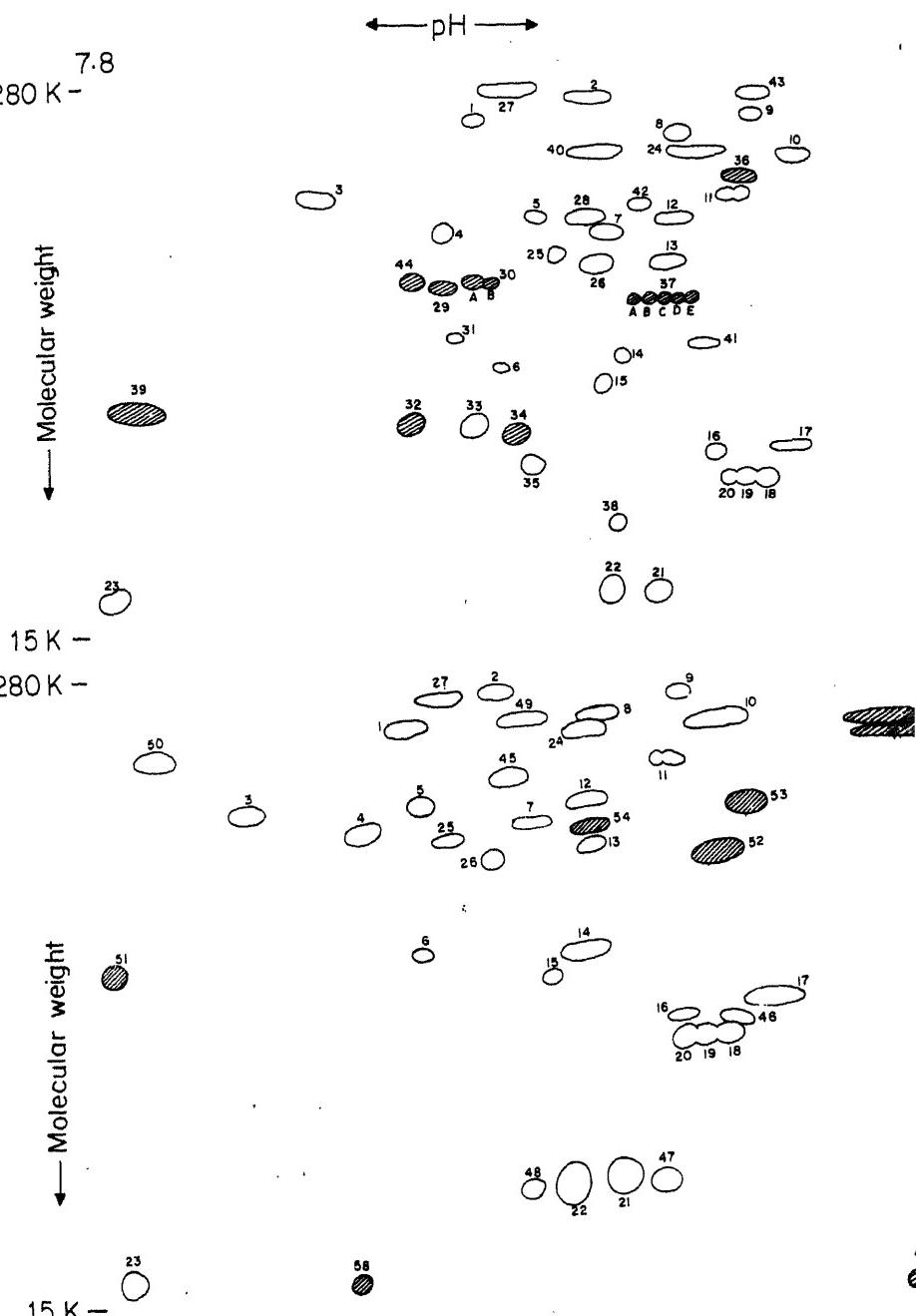
**Figure 2.** Tracings of radioautographs for comparing two dimensional gels: For convenience of comparison the radioautograph is divided into six segments. Spots common to brain and body wall were circled and numbered arbitrarily from 1 to 534. A, brain; B, body wall. The total number of common spots is 517 as 17 spots initially numbered turned out to be uncommon. Corresponding spots from brain and body wall carry identical numbers. A common spot is not necessarily present in every gel e.g. spot 406 and 485 above (see text for definition of common spots).



**Figure 3.** Distribution of spots unique to brain and body wall: A-brain and B-body wall. 61 unique spots in the brain gel and 66 unique spots in the body wall gel are cross hatched. Segments of which enlargements have been shown in Figure 4 are marked as insets.



**Figure 4.** Segmental enlargements of selected regions of brain and body wall gels showing the unique spots: Arrows show the presence of a unique spot in gels of one type and its absence in a corresponding position in gels of the other type. A, B, and C represent the segments of brain and body wall gels which are shown as insets in Figure 3.



**Figure 5.** Tracings of radioautographs showing Con-A binding glycoproteins in 2D gels: A - brain B - body wall. Common spots are numbered 1-27. Unique spots are cross hatched.

## Discussion

O'Farrel (1975) was able to detect over 1100 spots on two dimensional gels of *Escherichia coli* and similar numbers in *Caenorhabditis elegans*. The observed numbers were close to the estimated number of polypeptides in these organisms. Rodgers and Shearn (1977) found 330 spots in imaginal discs of *Drosophila* and 435 spots in whole larvae devoid of discs, labelled for short period. The 600 or so spots seen by us in uniformly labelled larval tissues are considerably short of the total number of sodium dodecyl sulphate peptides that might be expected on theoretical grounds (Judd *et al.*, 1972). This is not surprising. As pointed out by Rodgers and Shearn (1977), the technique used by us will fail to detect peptides which are insoluble in 9 M urea and NP40, peptides deficient in sulphur, peptides whose molecular weights lie beyond the range of 15,000-280,000 daltons or whose isoelectric points are beyond the chosen range of pH. Besides we have neglected extremely faint as well as erratic spots. The observed number of spots is nevertheless large enough to make the arduous task of comparing wild type and mutant patterns worthwhile.

A comparison of body wall and brain peptides allows us to estimate the proportion of tissue specific proteins. The 66 unique spots in body wall extracts and 61 in brain represent about 10% of the total. Although the proportion of tissue specific proteins in the two cases appears to be the same, the distribution of the spots across the gel is noticeably distinctive. The unique spots in brain gels are, more or less, uniformly distributed over the entire gel. The unique spots in body wall gels, on the other hand are clustered towards the alkaline end of the gel in the pI range of 7-7.5. It is likely that muscle specific proteins are among these spots.

Con-A-binding glycoproteins make up about 7% of larval proteins, and 64% of the glycoprotein spots are common to body wall and brain. 18% of brain glycoproteins (8 out of 44) and 18% of body wall glycoproteins (8 out of 41) are unique. The proportion of tissue specific spots among glycoproteins, thus appears to be higher than other proteins.

## Acknowledgements

Authors thank Dr K. S. Krishnan for helpful discussions, and Dr S. M. Joshi for technical assistance and instrumental modifications.

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## **Effect of copper intrauterine contraceptive device on carbohydrate metabolism in rat endometrium**

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MS received 3 January 1980; revised 15 April 1980

**Abstract.** Activities of phosphorylase, glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, malate dehydrogenase and succinate dehydrogenase in the rat endometrial tissue are significantly inhibited by an intrauterine copper device, while it stimulated glucose-6-phosphate dehydrogenase activity. The copper device decreased the lactate/pyruvate ratio in the tissue; pyruvate utilization *in vitro* by the rat endometrium is also blocked by copper. These findings suggested that the normal carbohydrate metabolism of the tissue may be affected in presence of copper, thus resulting in a change of the endometrial function, which may be one of the factors responsible for the contraceptive and pharmacological action of an intrauterine copper device.

**Keywords.** Intrauterine copper device; dehydrogenases; lactate/pyruvate ratio: carbohydrate metabolism; endometrial function.

### **Introduction**

Current opinion about the mechanism of action of an intrauterine copper contraceptive device suggests that copper induces a change in the biochemical composition of the uterus which interferes with the implantation of the fertilized ovum (Zipper *et al.*, 1969; Chang and Tatum, 1970; Hester *et al.*, 1970; Hicks *et al.*, 1975). In this context, an attempt has been made in this paper to assess the effect of intrauterine metallic copper on the carbohydrate metabolism by estimating the activity of a few enzymes of carbohydrate metabolism as well as the levels of lactate and pyruvate in the normal and copper-treated rat endometrium. In addition, the effect of metallic copper on the uptake of pyruvate by the rat endometrium *in vitro* was also studied.

### **Materials and Methods**

#### *Animals*

Healthy, postpubertal (100 day old) female virgin albino rats of nearly equal weight ( $160 \pm 15$  g) of the Institute colony and maintained under uniform husbandry conditions were used in this investigation.

#### *Experimental groups*

The animals were divided into three groups. In the first group (A) pieces of pure copper wire (20 mm length; 0.2 mm diameter;  $12.6 \text{ mm}^2$  area) were fitted bilaterally

into the uterine lumen of the animals. Similarly, in the second group (B) nylon sutures of the same length, area and diameter were fitted. The uterine horns of the animals of the third group (C) were sham operated to serve as control. All the surgical procedures were carried out under aseptic conditions.

#### *Collection of samples*

Thirty days after operation, the animals were sacrificed during the estrous phase and the uterine horns were dissected out. The endometrial scrappings were collected according to a previous procedure (Dasgupta *et al.*, 1972) and kept in isotonic sucrose solution prepared in glassdistilled water. The tissue was homogenized in a motor-driven all-glass homogenizer at a moderate speed for 10 min with interruptions in homogenization to prevent heating. The crude homogenate corresponding to a protein concentration of  $50 \pm 4$  mg/ml was centrifuged at 600 g to remove the nuclear fraction according to the method of Schneider (1948) and the supernatant fraction was used for the enzyme assays.

#### *Enzyme assay*

The activities of phosphorylase (EC 2.4.1.1), glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), lactate dehydrogenase (EC 1.1.1.27), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and malate dehydrogenase (EC 1.1.1.37) were determined by the procedures outlined in the Enzyme Manual of Worthington Biochemical Corporation, Freehold, NJ, USA (1971). The activity of succinic dehydrogenase (EC 1.3.99.1) was determined by the method of Cooperstein *et al.* (1950). Enzyme activities were also determined in presence of EDTA ( $10 \mu\text{mol}$ ).

#### *Estimation of lactate and pyruvate contents*

Deproteinised extracts of the tissue obtained after treatment with 10% (w/v) trichloroacetic acid were used for the estimation of lactate by the method of Hohorst (1963) and pyruvate by the method of Friedmann and Haugen (1943).

#### *In vitro uptake of pyruvate by rat endometrium*

The pyruvate consumption by the rat endometrium was estimated by incubating endometrial homogenate for 12 h at  $37^\circ\text{C}$  in the presence of metallic copper wire (20 mm length; 0.2 mm diameter;  $12.6 \text{ mm}^2$  area). Additionally, sodium pyruvate ( $10 \mu\text{mol}$ ) and sodium fumarate ( $5 \mu\text{mol}$ ) were added to the reaction mixture at 0 h. Under similar conditions, equal volume of the above homogenate was also incubated separately without the metal to serve as a control. Samples were drawn at intervals of 2 h for 12 h and the pyruvate content was measured (Friedmann and Haugen, 1943). Pyruvate consumption was also studied in presence of EDTA ( $10 \mu\text{mol}$ ).

#### *Protein determination*

Protein was estimated by the method of Lowry *et al* (1951) using bovine serum albumin as the standard.

#### *Statistical analysis*

A student's t-test was applied to determine the significance of the differences observed among the groups.

## Results

Table 1 shows the changes in the enzymic activity of the endometrium due to intrauterine nylon and copper devices. It was found that the activities of phosphorylase,

**Table 1.** Effect of intrauterine nylon and copper devices on enzyme activity of rat endometrium

Enzyme	Copper-treated (A)		Nylon treated (B)	Control (C)
	None	+EDTA		
Phosphorylase <sup>a</sup>	0.63±0.21 <sup>c</sup>	0.92±0.30	1.09±0.11	1.07±0.08
Glyceraldehyde-3-phosphate dehydrogenase	0.15±0.009 <sup>c</sup>	0.06±0.02	0.05±0.02	0.04±0.01
Lactate dehydrogenase <sup>b</sup>	0.85±0.12 <sup>c</sup>	1.02±0.02	1.15±0.04	1.17±0.05
Malate dehydrogenase <sup>b</sup>	0.53±0.22 <sup>c</sup>	1.00±0.05	1.00±0.06	1.08±0.05
Succinate dehydrogenase <sup>b</sup>	1.13±0.11 <sup>c</sup>	1.55±0.32	2.00±0.12	1.99±0.08
Glucose-6-phosphate dehydrogenase <sup>b</sup>	1.32±0.33 <sup>d</sup>	0.75±0.22	0.75±0.30	0.82±0.21

Mean values ±s.e.m. from 5 replicate estimations. Each estimation was done in triplicate in a tissue sample pooled from 4 animals.

<sup>a</sup> μg inorganic phosphate formed/mg protein per min.

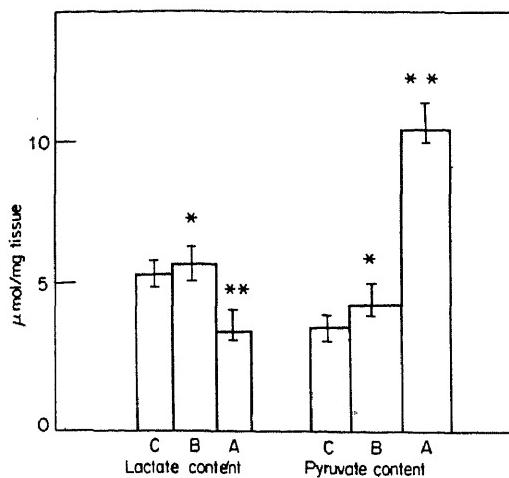
<sup>b</sup> Change in absorbance/mg protein per min.

<sup>c</sup> Significantly different from Groups B and C. P < 0.01

<sup>d</sup> Significantly different from Groups B and C. P < 0.05.

glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, succinate dehydrogenase and malate dehydrogenase recorded a significant inhibition (P < 0.01) in the group A as compared with Groups B and C. On the other hand, the activity of glucose-6-phosphate dehydrogenase showed a significant enhancement (P < 0.05) in the Group A. However, a nylon suture was found to be ineffective in this respect and the values in Group B were comparable with those of Group C. Further EDTA, a metal chelator, seemed to suppress the action of copper and no change in the activity of enzymes was observed in its presence.

Figure 1 summarizes the results showing the effect of intrauterine nylon and copper sutures on the levels of lactate and pyruvate in the rat endometrium. It was found that the concentration of lactate decreased whereas that of pyruvate increased (P < 0.05) in the Group A as compared with Groups B and C. However, in the nylon treated group, no change in the lactate/pyruvate content was observed and the values of Group B were comparable with those of the Group C.

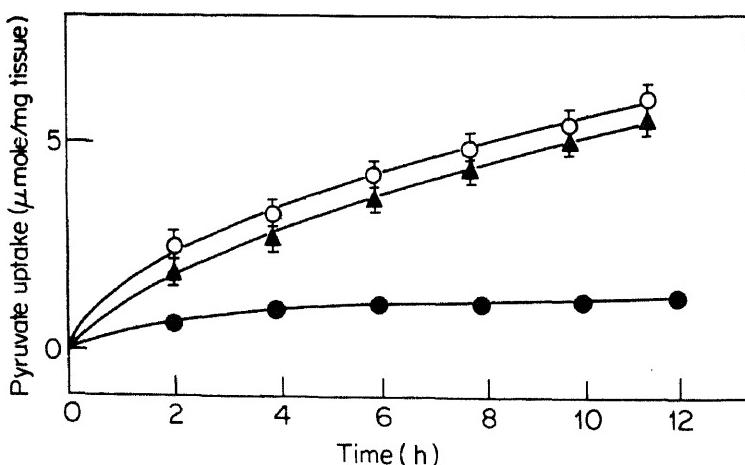


**Figure 1.** Effect of intrauterine nylon and copper devices on the lactate and pyruvate contents in the rat endometrium. Columns represent mean values and vertical bars  $\pm$ s.e.m. from 5 replicate estimations. Each estimation was done in triplicate in a tissue sample pooled from 4 animals. A=copper treated, B=nylon-treated and C=control.

\* Non-significant as compared with Group C

\*\* Significantly different from Groups B and C-P < 0.05.

Figure 2 reveals the effect of metallic copper on the utilization of pyruvate by the rat endometrium. It was noted that the metal significantly blocked the pyruvate uptake by the tissue. This effect was not observed when EDTA was added in the reaction mixture.



**Figure 2.** Effect of copper on the pyruvate uptake by the rat endometrium. Each point represents mean value  $\pm$  s.e.m. from 5 estimations done in duplicate in different tissue samples each pooled from 4 animals. 5 ml endometrial homogenate, corresponding to  $50 \pm 4$  mg protein/ml was incubated under the conditions specified in the Materials and Methods section. Tissue was allowed to respire for 1 h to exhaust the endogenous pyruvate before the experiment was started control, O, control+Cu, ●; control+Cu+EDTA, ▲.

### Discussion

The efficacy of a Copper-IUCD (copper intrauterine contraceptive device) has been associated with various chemical reactions; the metal may trigger in the uterus (Oster 1971 and 1972). In this regard it may be mentioned that a consistent absorption of copper and its distribution in different organs including uterus has been shown to occur within hours after insertion of the device (Okereke *et al.*, 1972; Moo-Young *et al.*, 1973; Salaverry *et al.*, 1973; Moo-Young and Tatum 1974; Ranney *et al.*, 1975), thus disturbing the normal concentrations of the copper in the cell (Hernandez *et al.*, 1975). Copper is a well known enzyme poison and accumulation of large amounts of the metal in cells would sooner or later lead to enzyme inhibition and tissue necrosis (Walshe, 1960). Copper actively interacts with free sulphhydryl and disulphide groups, depending upon the chemical characteristics of the environment (Klotz, *et al.*, 1952). Both kind of reactive functional groups play an indispensable role in the spatial structure and in the active site function of some key enzymes (including those studied in this work) involved in the utilization of hexoses in the tissue (Bover, 1959). An alteration in the activities of such enzymes caused either by chelation with copper ion or oxidation of SH groups (Klotz *et al.*, 1952) can occur in the copper treated rat endometrium. This suggestion is further reinforced by the observation that copper-induced changes in the enzymic activity is no longer seen in the presence of EDTA, a well-known metal chelator.

Inhibition of phosphorylase would cause a decrease in the conversion of glycogen to lactate. Besides, a decrease in the level of lactate with the concomitant increase of pyruvate and a drastic decrease in the lactate/pyruvate ratio may also be attributed to an inhibition of lactate dehydrogenase. Earlier, pyruvate and lactate have been shown to be essential for the maintenance of developing mouse embryo; pyruvate being more essential for maintaining the activity of tricarboxylic acid cycle (Quinn and Wales, 1973; Wales and Whittingham, 1973). Thus it appears that an intrauterine copper device may disturb the critical lactate/pyruvate ratio of the endometrial tissue and render it metabolically deficient.

In the event of an inhibition of glyceraldehyde-3-phosphate dehydrogenase activity, the utilization of glucose through Embden-Meyerhoff pathway may be severely handicapped. Earlier, metallic copper has been shown to reversibly inhibit muscle glycolysis (Lipmann, 1937). Partial inhibition of succinate and malate dehydrogenase would affect the functionality of tricarboxylic acid cycle. This is further reinforced by the observed decrease in the pyruvate utilization by the rat endometrium in the presence of metallic copper. In fact, copper has been found to block the entry of pyruvate into the pigeon brain by interacting with lipoic acid (Peters and Walshe, 1966) and membrane adenosine triphosphatase (Peters *et al.*, 1966).

These findings draw our attention to the fact that if intrauterine copper device would suppress the normal energy producing pathways, then the tissue must obtain a supply of energy by some other route. One possibility is that the pentose phosphate pathway may be involved and in the process producing reduced coenzymes in the form of NADPH. The reduced nicotinamide nucleotides are known to be coupled by transhydrogenase mechanisms normally found in the mammalian tissue (Kaufman and Kaplan, 1961). Therefore, it seems possible that a cell may survive the presence of an agent that partially blocked the tricarboxylic acid cycle if it possessed more than normally active pentose phosphate pathway. Our results show that glucose-6-ph-

phate dehydrogenase, an oxidative enzyme of pentose phosphate pathway appears to be stimulated in the presence of intrauterine copper. This suggests that in copper treated endometrial tissue, metabolic flux may be altered from usual glycolysis to pentose phosphate pathway. Such an event would also increase the aerobicity of the uterine milieu which was suggested to be unfavourable for at least one process, namely fertilization (Gross, 1961), although, intrauterine copper does not affect this process (Middleton and Kennedy, 1975).

Pentose phosphate pathway has also been associated with phagocytizing polymorphonuclear leucocytes (Paul and Sbarra, 1968) accumulation which was suggested to be involved in the pharmacological action of intrauterine devices (Cuadros and Hirsh, 1972).

Thus the present findings suggest that copper may affect the normal carbohydrate metabolism of the tissue, which may result in the alteration, in more than one way, of the endometrial function of the uterus, presenting an unfavourable environment for the process of implantation.

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## **Immunochemical relationship between glucoamylases I and II of *Aspergillus niger***

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MS received 30 May 1980; revised 21 July 1980

**Abstract.** Rabbit antisera were prepared against the purified glucoamylases I and II of *Aspergillus niger*. Relationships between the two enzyme forms were investigated by using the antisera in immunodiffusion and immunoinhibition experiments. Both the forms of glucoamylase gave a single continuous precipitin band demonstrating very close structural resemblance. They gave almost identical immunoprecipitation patterns and had the same equivalence points indicating that the two forms of *A. niger* glucoamylases were immunologically identical. The enzyme treated with periodate was immunologically identical with the controls and had slightly less enzyme activity but showed greatly reduced stability on storage at 4°C.

**Keywords.** Glucoamylases; immunochemical relationship; *Aspergillus niger*.

### **Introduction**

Two forms of glucoamylases (I and II) (EC 3.2.1.3) occur in *Aspergillus niger* and their properties have been described (Pazur and Ando, 1959; Pazur and Kleppe, 1962; Fleming and Stone, 1965; Lineback *et al.*, 1969; Pazur *et al.*, 1971; Venkatramu *et al.*, 1975; Manjunath and Raghavendra Rao, 1979). These enzymes possess many similar properties such as the pH and temperature optima, action pattern on oligosaccharides and ability to cleave  $\alpha$ -1,4;  $\alpha$ -1,6; and  $\alpha$ -1,3 bonds between glucose moieties, amino terminal amino acid, glycoprotein nature etc. On the other hand, detailed examination of the literature shows many disparate properties of glucoamylases I and II, such as molecular weight, electrophoretic mobility, amino acid content and carbohydrate content. However, none of these properties by itself can differentiate these two enzymes. Immunological tests can apparently distinguish related proteins (Aw and Hobbs, 1968; Dingle *et al.*, 1971; Carroll and Robinson, 1972; Srivastava and Bentler, 1972; Matsuzaki *et al.*, 1974; Messer and Dean, 1975; Phillips *et al.*, 1975; Hayashi and Nakamura, 1976). It was therefore of interest to study the immunochemical behaviour of these two enzymes to elucidate structural relationships. In this paper, we report the results of such a study using the techniques of immuno-double diffusion and immunoprecipitation.

### **Materials and Methods**

The materials used in the study were procured from the following sources: Glucozyme (glucoamylases from *A. niger*) from Anil Starch Products, Ahmedabad; Noble Agar

and Freunds's Complete Adjuvant from the Difco Laboratories, Detroit, MI, USA; soluble starch from the British Drug House, Bombay; glucose oxidase and peroxidase from Sigma Chemical Company, St. Louis, MO, USA; o-dianisidine from the CSIR Centre for Biochemicals, V.P. Chest Institute, Delhi. All other reagents used were of analytical grade unless otherwise stated.

Homogenous preparations of glucoamylases I and II were obtained according to the procedure described previously (Manjunath and Raghavendra Rao, 1979).

*Glucoamylase activity* was assayed as described earlier (Manjunath and Raghavendra Rao, 1979) using soluble starch as substrate. Protein was estimated according to the procedure of Lowry *et al.* (1951) using bovine serum albumin as the standard. One unit of glucoamylase releases one micromol of glucose per min under assay conditions.

*Preparation of antisera.* The rabbits were bled before immunization to provide control sera. The glucoamylase solution containing 10 mg of protein in saline was emulsified with an equal volume (5 ml) of complete Freund's Adjuvant. Rabbits were given intracutaneous or intramuscular injections of the antigen (1 ml) at multiple sites at 10-day intervals over a period of 50 days, and were bled from the marginal ear vein 15 days after the last injection. The blood was allowed to clot at 25°C for 2 h and the serum was collected by centrifugation at 3000 g for 10 min. Antisera A and B were derived from rabbits which received homogenous preparation of glucoamylases I and II isolated from Glucozyme, respectively. Antiserum C was derived from a rabbit which received injections of a dialysed aqueous extract of Glucozyme. Control sera and antisera were stored at -20° in presence of 0.2% sodium azide as preservative.

*Immuno-double diffusion analysis* by the method of Ouchterlony (1967) was performed at 4°C by using 20  $\mu$  l capacity wells in 1.5% agar in 20 mM KH<sub>2</sub>PO<sub>4</sub>-Na<sub>2</sub>HPO<sub>4</sub> buffer, pH 7.6 containing 0.15 M NaCl and 0.01% sodium azide. After 16-20 h of diffusion, white precipitin bands became visible and plates were photographed under dark ground illumination (Ouchterlony and Nilsson, 1973).

*Immunoelectrophoresis* was performed at pH 8.6 as described by Dean (1974). Immunoelectrophoresis plates (8.4×10 cm) were coated with 12 ml of 1.5% agar in 0.05 M Veronal buffer pH 8.6 containing 0.01% sodium azide. The antigen well had a capacity of 5  $\mu$  l and the antiserum trough of 80  $\mu$  l. Electrophoresis was carried out for 6 h at 300V, 10 mA and immunodiffusion was carried out at 4°C for 2 days to permit complete formation of precipitin lines. The plates were photographed under dark ground illumination (Ouchterlony and Nilsson, 1973).

*Immunoprecipitation.* A constant amount (100  $\mu$  l) of suitably diluted enzyme (0.1 mg/ml) was placed in a series of test tubes (10×0.5 cm). Then antiserum (0-100  $\mu$  l) was added to the tubes and the volume made up to 500  $\mu$  l with 20 mM phosphate buffered (0.15 M) saline pH 7.0. After incubation at 37°C for 2 h, the tubes were stored at 4°C for 36 h (control experiments for various time-intervals showed that incubation was maximum after 36 h) and centrifuged at 1500 g for 15 min. The supernatant was removed and 100  $\mu$  l assayed for glucoamylase activity by the normal procedure. The equivalent points were calculated by plotting the activity remaining in the supernatant against the volume of the antisera in the incubation mixture.

A control experiment showed that the antisera had no glucoamylase activity. In further control experiments with normal rabbit serum in place of antiserum, no immuno inhibition of glucoamylase activity was observed.

*Periodate treatment.* Samples of enzyme (4 mg/ml) were separately treated in the dark with 0.1 M NaIO<sub>4</sub> in 0.05 M sodium acetate buffer (pH 4.5) at 25°C for 20 min, 1 h and 2 h. The reaction was stopped by the addition of a 5-molar excess of thioglycollate and the reaction mixture passed through a Biogel-P 6 (20×0.5 cm) column equilibrated with 0.05 M sodium acetate buffer pH 4.8. The enzyme was eluted by the same buffer. It was then stored at 4°C and the activity periodically determined.

## Results

Glucoamylases I and II of Glucozyme (*A. niger* glucoamylase) were purified by the method described elsewhere (Manjunath and Raghavendra Rao, 1979). The final, freeze-dried products, glucoamylases I and II were found to be homogeneous by ultra centrifugation and by polyacrylamide gel electrophoresis methods and had specific activities of 86 and 91 units respectively.

*Immunoelectrophoresis and purity.* The purity of the glucoamylases was also tested by immunoelectrophoresis against polyspecific antiserum C (raised against dialyzed aqueous extract of Glucozyme) and antisera A and B. Antiserum C was found to precipitate at least five proteins in the crude Glucozyme extract. With antisera A and B, a single precipitin line was observed on immunoelectrophoresis of the homogenous enzymes (figure 1). Thus by immunoelectrophoresis also, glucoamylases I and II were found to be homogeneous.



**Figure 1.** Immunoelectrophoresis of purified preparations of glucoamylases I (a) and II (c) and crude glucozyme extract (b) using polyspecific antiserum C (raised against crude-glucozyme extract).

*Immunoprecipitation.* The immunoprecipitation-inhibition patterns are shown in figure 2. The precipitation curves for glucoamylases I and II with antiserum A

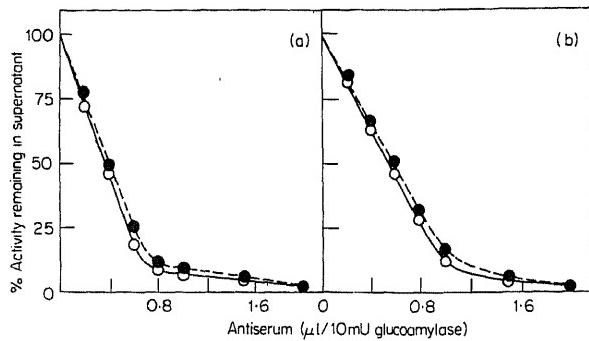


Figure 2. Immunoprecipitation curves of glucoamylases I and II with antiserum A (a) and antiserum B (b). Glucoamylase I, ●; Glucoamylase II, ○.  
Experimental details of assay procedure are described in materials and methods.

were identical (figure 2a). The equivalence points for glucoamylases I and II, were  $71 \mu\text{l}$  and  $69 \mu\text{l}$  antiserum/unit of enzyme activity respectively. Precipitation curves obtained with antiserum B were also similar (figure 2b). The equivalence points with this antiserum for glucoamylases I and II were  $109 \mu\text{l}$  and  $108 \mu\text{l}$  of antiserum/unit of enzyme activity, respectively.

The supernatant obtained by centrifugation ( $10,000 \text{ g}$ ; 10 min) of a reaction mixture containing glucoamylase I and its equivalent or slightly less of antiserum A does not inhibit glucoamylase II. In converse experiments, no inhibition of glucoamylase I was noticed. Hence the antisera A and B did not appear to contain antibodies specific only for either glucoamylase I or glucoamylase II.

*Ouchterlony immuno-double diffusion.* The immuno-double diffusion patterns of glucoamylases I and II against antisera A and B are shown in figure 3. In both

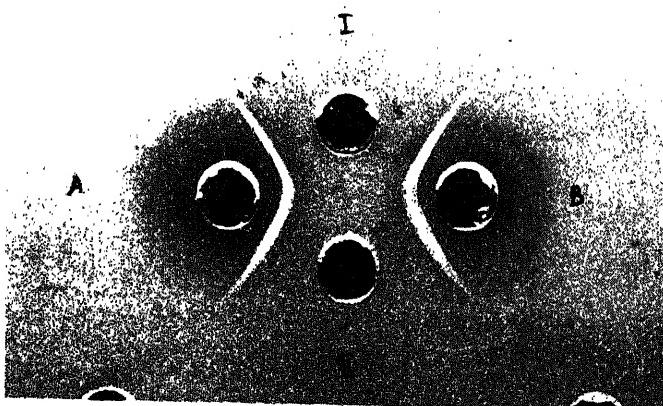


Figure 3. Ouchterlony immuno-double diffusion of glucoamylases I and II.  
A, Antiserum A; B, Antiserum B.

instances, only one sharp precipitin line was obtained which was continuous in adjacent diffusions of glucoamylases I and II; there was no evidence of spur formation.

*Effect of treatment with periodate.* Whether the carbohydrate moiety was essential for antigenic and enzyme activity and for stability was checked by periodate treatment. The results are shown in figure 4 (a, b and c). Even when 75% of the carbohydrates of the glycoprotein enzyme were stripped, enzyme activity and antigenic behaviour were not affected, but the storage stability at 4°C was greatly reduced.

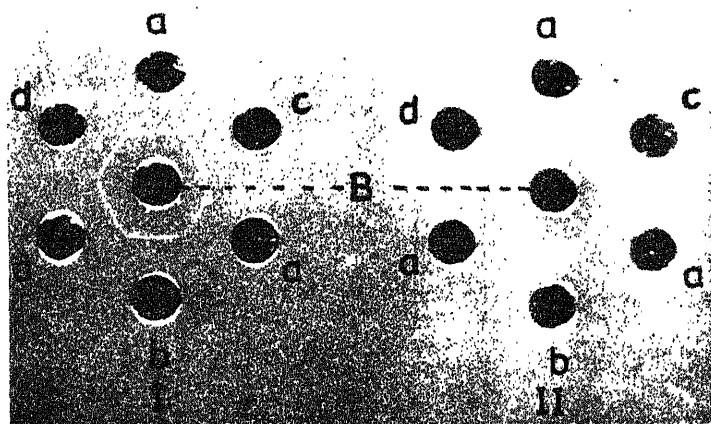


Figure 4. Immunological behaviour of the periodate-treated glucoamylases I and II.

(a) Immuno-double diffusion pattern of a, native enzyme and b, c and d, enzyme treated with periodate for 20, 40 and 60 min respectively. B —— antiserum for glucoamylase II.

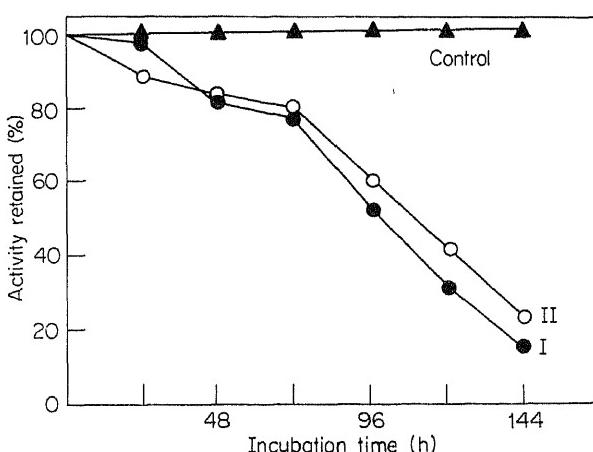
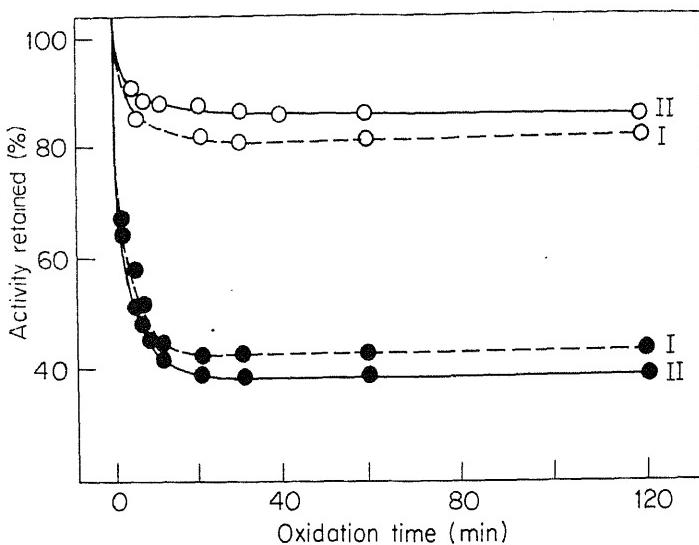


Figure 4. (b). Activity (○) and carbohydrate (●) content of glucoamylase after treatment with periodate.



**Figure 4.** (c). Storage stability of glucoamylases I and II treated with periodate for 20 min. Aliquot samples of the enzyme stored at 4°C were withdrawn at time periods indicated in the figure and assayed for activity. An enzyme sample not treated with periodate but stored at 4°C served as the control. Activity of the control was normalized to 100 and per cent activity retained was plotted against time of storage.

## Discussion

Immunoprecipitation has been shown to be a very sensitive technique to study the immunological relationships of closely related forms of enzymes. Examples are human liver hexosaminidases A and B (Carroll and Robinson, 1972; Srivastava and Bentler, 1972), chicken liver cathepsin D isoenzymes (Dingle *et al.*, 1971), human liver  $\alpha$ -mannosidase A and B (Phillips *et al.*, 1975), bacterial amylases (Matsuzaki *et al.*, 1974), human amylases (Aw and Hobbs, 1968), rat  $\alpha$ -amylases (Messer and Dean, 1975) and fungal glucose oxidase (Hayashi and Nakamura, 1976). In the present investigation, this technique as well as immunodiffusion were used to find out the immunological identity/disparateness of the two forms of *A. niger* glucoamylases.

Precipitation of purified glucoamylase I by antiserum B and precipitation of purified glucoamylase II by antiserum A showed that the two forms of glucoamylases were very closely related. Further, the similarity of their equivalence points and the lack of spur formation (figures 3 and 4a) in the continuous precipitin line obtained when purified glucoamylases I and II were placed in adjacent wells in an immunodiffusion experiment suggest that they are immunologically identical. The absence in the antiserum of antibodies very specific for either glucoamylase I or II supports this conclusion. The differences noticed in the immunological equivalents (figure 2) are perhaps due to the differences in the concentration of the antibodies in antisera A and B. But the equivalents seem identical for both forms of the enzyme with either antiserum A or B. Carbohydrates are known to be antigenic, e.g., capsular

polysaccharides of *Pneumococci*. But in the present instance, the carbohydrate moiety of the glucoamylases do not seem to have a role in the antigenicity of the enzyme, since the removal of as much as 75% of the carbohydrate had no effect on the immunological behaviour or significantly on the activity of the enzyme: however, categoric statements regarding this cannot be made yet since the periodate treated enzyme still contained much carbohydrate (25% of the original). As other experiments have indicated, the carbohydrate moiety seems to stabilize the enzyme as seen in the studies on the storage of periodate treated enzyme.

Glucoamylases I and II of Glucozyme, which are easily separable from one another, differ in a number of physical and chemical properties (Manjunath and Raghavendra Rao, 1979). Yet the fact that they are immunologically "identical" according to the tests conducted, is unexpected. The possibility that the two forms might arise as artifacts due to proteolysis or deamidation has been considered. But this has been deemed very improbable because of many facts such as the absence of proteolytic activity in the crude enzyme powders, the mild procedural conditions during the preparation of the enzymes, very similar molecular weights and specific activities but fairly significant differences in composition with respect to carbohydrate and amino acids and each form of enzyme being present in considerable proportion (I:II 3:7). Therefore the results in the present investigation indicate that there is a close structural and functional relationship between the two forms of glucoamylases in *A. niger* (Glucozyme).

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## A comparative study of 5'-nucleotidase and alkaline phosphatase in human placenta during development

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**Abstract.** Activities and a few properties of alkaline phosphatase and 5'-nucleotidase were compared in the developing human placenta. Both the enzymes were mostly membrane-bound and displayed similar developmental patterns with the highest activities at 24/26 weeks of the placenta. L-Phenylalanine, L-tryptophan and L-leucine were inhibitors of alkaline phosphatase, whereas they had no effect on the 5'-nucleotidase. Alkaline phosphatase from a late stage of gestation appeared to be almost heat-stable. An appreciable part of 5'-nucleotidase was also resistant to heat inactivation and this fraction varied with gestational age of the tissue. For both the enzymes,  $V_{max}$  changed without altering  $K_m$  values with periods of gestation.  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{Mn}^{2+}$  ions stimulated the alkaline phosphatase activity and  $\text{Hg}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Ni}^{2+}$  were inhibitory. 5'-Nucleotidase was not activated by any of these cations. EDTA and Concanavalin A inhibited both the enzymes, although the extent of inhibition was different and also varied with gestation.

**Keywords.** Human placenta; alkaline phosphatase; 5'-nucleotidase.

### Introduction

5'-Nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) is widely distributed in living systems (Bodansky and Schwartz, 1968) including human placental tissues (Hayashi *et al.*, 1964; Krishnanantha and Maguir, 1978). The presence of phosphomonoesterases with peak activity at alkaline pH (Orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) is a distinguishing feature of syncytial trophoblast of the placenta (Fishman *et al.*, 1976; Sugiura *et al.*, 1977). The activity of non-specific phosphatases interfered with the evaluation of 5'-nucleotidase activity (Bodansky and Schwartz, 1968). In an attempt to circumvent this difficulty, it seemed worthwhile to study the properties of these two enzymes in human placental tissues. We have, therefore, conducted a comparative study of 5'-nucleotidase and alkaline phosphatase throughout the gestational ages of human placenta and a method for assaying 5'-nucleotidase is reported.

## Materials and methods

### Chemicals

*p*-Nitrophenyl phosphate, 5' AMP and bovine serum albumin were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. Concanavalin A was a gift from Dr B. K. Bachhawat, Indian Institute of Experimental Medicine, Calcutta. All other chemicals used were of analytical grade and were purchased locally.

### Biological Material

Human placental tissues were collected from the Department of Obstetrics and Gynaecology, S.S.K.M. Hospital, Calcutta. Term placentas were obtained from normal term deliveries and other tissues of different gestational ages were collected from women undergoing legal abortion either by suction or by hysterotomy. Gestational age was determined from the period of amenorrhea and by the size, weight and crown-rump length of the fetus. This method generally provides data correct to  $\pm$  one week (Kaplay, 1976). The placenta were kept on ice until processing.

### Preparation of the enzymes

The cross-sectional pieces of the tissues were taken, chopped and washed with ice-cold distilled water to free it from blood as far as practicable. The tissues were homogenized in a Potter-Elvehjem glass homogenizer at 0-4°C in 0.25 M sucrose to make a 10% (w/v) tissue concentration. The cell debris and nuclear residues were removed by centrifugation in the cold (0-4°C) at 1500 g for 10 min in an International Refrigerated centrifuge (Model B-20). The supernatant fluid was then recentrifuged at 12,000 g for 30 min for isolation of the mitochondria. The supernatant thus obtained was centrifuged at 105,000 g for 1 h in a Spinco Ultracentrifuge, Model L. The pellet was used as the microsomal fraction and the supernatant as the cytosol fraction. The microsomal pellet was suspended in ice-cold 0.25 M sucrose. This suspension, unless otherwise stated, was used as the enzyme preparation for the study of 5'-nucleotidase and alkaline phosphatase.

### Enzyme assay

5'-Nucleotidase activity was measured in a reaction mixture containing 80 mM Tris-HCl, pH 7.5; 0.5 mM 5' AMP, 30-40  $\mu$ g of the enzyme and with or without 20 mM L-tryptophan (a potent inhibitor of alkaline phosphatase) in a total volume of 1.0 ml. Following a 30 min incubation at 37°C, 0.25 ml of ice-cold 30% trichloroacetic acid was added. The samples were then chilled in ice and centrifuged for 10 min at 2000 g. The phosphate content ( $P_i$ ) in the supernatant was determined by the method of Lowry and Lopez (1946). The amount of  $P_i$  liberated/mg protein/h was considered as the specific activity of 5'-nucleotidase.

For the estimation of alkaline phosphatase, the assay mixture containing 1 mM of *p*-nitrophenylphosphate, 80 mM Tris-HCl, pH 9.6 and 30-40  $\mu$ g of the enzyme preparation was incubated at 37°C for 30 min. The reaction was stopped by the addition of 0.1 N NaOH and the formation of *p*-nitrophenol was measured colorimetrically in a Spectronic 20 spectrophotometer at 420 nm. The specific activity of alkaline phosphatase was defined as the amount of *p*-nitrophenol liberated/mg protein/h.

**Estimation of protein**

(mg/mg of protein)

Protein was estimated by the method of Lowry *et al.*, (1951) using crystalline bovine serum albumin as the standard.

**Results**

Table 1 summarizes the enzyme activities in the microsomal and supernatant fractions of human placenta with increasing periods of gestation. Both the enzymes were detected in human placenta as early as the 6th week of gestation. The enzyme

**Table 1.** Activity of alkaline phosphatase and 5'-nucleotidase in human placenta with increasing periods of gestation.

Gestation (weeks)	5'-nucleotidase <sup>a</sup> ( $\times 10^4$ )		Alkaline phosphatase <sup>b</sup> ( $\times 10^4$ )	
	Microsome	Cytosol	Microsome	Cytosol
6-8	12.59±1.02	0.86±0.08	1.02±0.12	0.26±0.03
10-12	15.92±1.10	1.31±0.10	3.00±0.17	0.35±0.03
14-16	18.02±1.39	1.42±0.11	4.05±0.20	0.59±0.06
18-20	21.97±1.63	1.64±0.13	8.17±0.83	1.40±0.12
22-26	23.40±1.98	2.12±0.19	15.73±1.21	3.00±0.16
36-40	20.79±1.60	2.65±0.20	14.91±0.98	3.66±0.28

The results are mean ± SEM of 6 observations in each case.

<sup>a</sup> Specific activity of 5'-nucleotidase is expressed as  $\mu\text{mol P}_i$  liberated/mg protein/h.

<sup>b</sup> Specific activity of alkaline phosphatase expressed as  $\mu\text{mol } p\text{-nitrophenol}$  liberated/mg protein/h.

activities in the microsomal fraction increased with the gestational age until 24/26 weeks (the latest stage studied). The activities, however, slightly declined at term. The supernatant fraction maintained a steady increase in activity with the advancement of pregnancy.

Table 2 compares the extent of inhibition of placental alkaline phosphatase and 5'-nucleotidase by L-phenylalanine, L-tyrosine and L-leucine. Alkaline phosphatase was strongly inhibited by these amino acids and the sensitivity gradually decreased with placental maturity. On the other hand, these amino acids had no effect on 5'-nucleotidase activity and this property was utilized in determining placental 5'-nucleotidase activity in the present study.

**Table 2.** Effect of amino acids on alkaline phosphatase and 5'-nucleotidase from the developing human placenta.

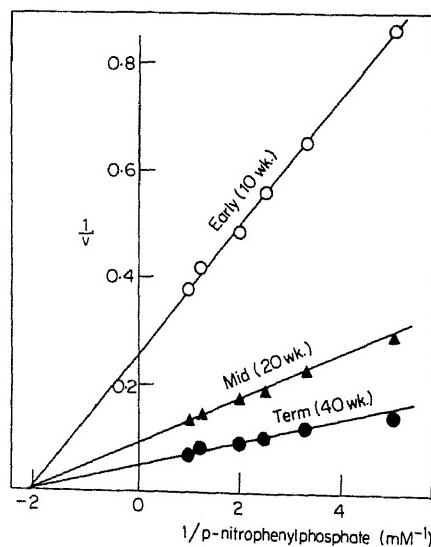
Amino acid used (mM)	inhibition (%)		
	Early (10 week)	Mid (20 week)	Term (40 week)
L-Phenylalanine (1)	45 (1)	26 (1)	19 (0)
L-Phenylalanine (5)	77 (2)	64 (2)	56 (2)
L-Tryptophan (1)	54 (2)	36 (1)	29 (1)
L-Tryptophan (5)	90 (2)	77 (2)	70 (2)
L-Leucine (1)	29 (0)	20 (0)	14 (0)
L-Leucine (1)	60 (1)	47 (1)	40 (0)

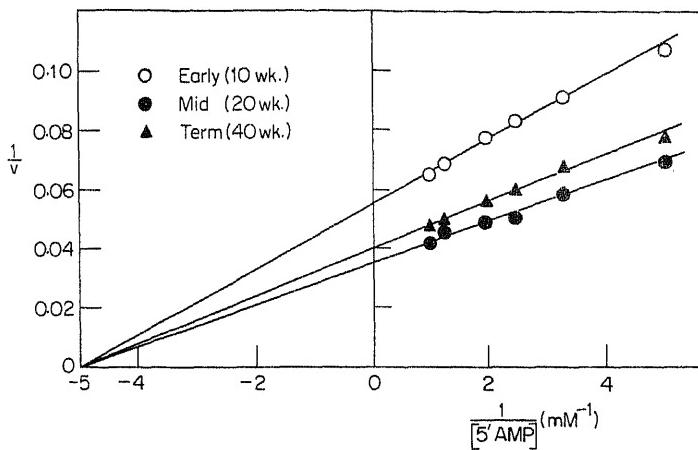
The incubation medium contained L-amino acid (1 mM or 5 mM) and the control system contained the corresponding D-amino acid. Percentage inhibition was calculated from  $(D-L) \times 100/D$

Figures in parenthesis indicate the percentage inhibition of 5'-nucleotidase.

The results are the average of 3 observations for each gestation period.

Lineweaver-Burk plots for alkaline phosphatase and 5'-nucleotidase during different stages of gestation are shown in figures 1 and 2 respectively. In both the cases,  $K_m$  values remained constant, while  $V_{max}$  changed during placental development.





**Figures 1 and 2.** Double reciprocal plots of the velocity against substrate concentration in different gestation of placenta. v—represents velocity expressed as specific activity.

Table 3 presents the kinetic parameters of *p*-nitrophenylphosphate hydrolysis in the absence and presence of inhibitors. The kinetic studies revealed that these amino acids altered both the  $K_m$  and  $V_{max}$  values of *p*-nitrophenylphosphate hydrolysis throughout the gestational ages.

**Table 3.** Kinetic parameters of alkaline phosphatase with inhibitors.

Treatment (mM)	$K_m$ (mM)	$V_{max}^a$ $\times 10^4$	$K_m$ (mM)	$V_{max}^a$ $\times 10^4$
	Early gestation (10 weeks)		Term placenta (40 weeks)	
Control	0.48	3.92	0.48	20.22
L-Phenylalanine (5)	0.10	0.77	0.22	10.00
L-Tryptophan (5)	0.04	0.34	0.11	5.26
L-Leucine (5)	0.18	1.47	0.28	12.90

<sup>a</sup>  $\mu$  mol *p*-nitrophenol liberated/mg protein/h.

Table 4 shows the thermostability (at 65°C for 10 min) of the enzymes. Alkaline phosphatase in early gestation was heat-labile. However, the enzyme was almost completely heat-stable as the pregnancy advanced. In contrast, the 5'-nucleotidase was stable during early period but the stability decreased during placental maturity.

**Table 4.** Effect of heat-treatment on alkaline phosphatase and 5'-nucleotidase from developing human placenta.

Gestation (in weeks)	Residual activity (%)	
	Alkaline phosphatase	5'-Nucleotidase
Early (8-10)	10	84
Mid (20-24)	99	64
Term (40)	99	47

For the study of heat-stability, the enzyme preparation was incubated at 65°C for 10 min, immediately cooled in ice, assayed for the remaining enzyme activity and compared with the control. The activity of the control was normalized to 100 and the residual activity in each case was expressed as per cent of this control value.

The results are the mean of 3 observations for each gestation.

The hydrolysis of *p*-nitrophenyl phosphate by alkaline phosphatase from the early placenta was activated by  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{Mn}^{2+}$ , but was strongly inhibited by  $\text{Hg}^{2+}$  and  $\text{Zn}^{2+}$ , while  $\text{Cu}^{2+}$ ,  $\text{Ni}^{2+}$  and  $\text{Co}^{2+}$  caused only slight inhibition (table 5). The enzyme in placenta obtained at term was more resistant and was inhibited only by  $\text{Hg}^{2+}$  and  $\text{Zn}^{2+}$ . On the other hand, the metal ions tested did not

**Table 5.** The Effect of metal ions and EDTA on 5'-nucleotidase and alkaline phosphatase from human placenta

Metal ion added	Alkaline phosphatase		5'-Nucleotidase	
	Early placenta	Term placenta	Early placenta	Term placenta
None	100	100	100	100
$\text{Hg}^{2+}$	0	15	2	12
$\text{Ca}^{2+}$	136	116	95	98
$\text{Mg}^{2+}$	116	112	101	106
$\text{Mn}^{2+}$	120	112	41	97
$\text{Co}^{2+}$	91	99	98	100
$\text{Zn}^{2+}$	0	17	7	25
$\text{Cu}^{2+}$	76	93	81	82
$\text{Ni}^{2+}$	80	111	79	100
EDTA (1 mM)	49	78	79	90

The activity in the absence of any added metal ion was normalized to 100 and the activity in the presence of these metal ions is expressed as per cent of this normalized acitivity.

The incubation medium contained the respective metal ion as its chloride salt to make a final concentration of 1 mM.

The results are the mean of 3 observations in each case.

stimulate the 5'-nucleotidase activity. While  $Hg^{2+}$ ,  $Zn^{2+}$  and  $Cu^{2+}$  inhibited the enzyme activity,  $Ni^{2+}$  and  $Mn^{2+}$  caused only marginal inhibition in early gestation, but not in term placenta.  $Ca^{2+}$ ,  $Mg^{2+}$  and  $Co^{2+}$  were without effect.

The extent of inhibition by EDTA of both the enzymes decreased as the age of the placenta increased.

Table 6 shows the effect of Concanavalin A on the enzyme activities. It is observed that Concanavalin A inhibited 5'-nucleotidase in early placenta to a greater extent

**Table 6.** Effect of Concanavalin A on 5'-nucleotidase and alkaline phosphatase from human placenta.

Concanavalin A added (in $\mu g$ )	Inhibition (%)			
	5'-Nucleotidase		Alkaline phosphatase	
	Early placenta	Term placenta	Early placenta	Term placenta
10	2	0	2	2
50	5	0	6	5
100	20	11	14	7
200	35	21	16	6
300	32	20	16	6

The results are the mean of 3 observations in each case.

than that in term placenta. The alkaline phosphatase behaved similarly, although the inhibition by the lectin was to a lesser extent.

## Discussion

The much higher activities of alkaline phosphatase and 5'-nucleotidase activities in the microsomal fraction compared to that in cytosol indicated that these enzymes were mostly membrane-bound. The steady increase in the enzyme activities in early gestation was a characteristic of tissues in active proliferation and the decreased activities at term possibly reflected the decreased physiological function and ageing of the tissue. Similar observations have also been reported with  $\alpha$ -glucosidase (Thanavala *et al.*, 1974), acid phosphatase (Kushari *et al.*, 1978), acetyl cholinesterase (Sastry *et al.*, 1976) and ATPase (Chakraborti and Mukherjea, 1980).

The inhibition of alkaline phosphatase by L-phenylalanine, L-tyrosine and L-leucine is consistent with the findings of several investigators (Ghosh and Fishman, 1966; Lin *et al.*, 1971; Nakayama *et al.*, 1970). The reason for the progressively decreased inhibition of alkaline phosphatase by amino acids with increase in gestation is still not clear. This observation, however, strengthens the suggestion that this enzyme is metabolically modified during development (Fishman *et al.*, 1976).

The measurement of 5'-nucleotidase activity can be affected by the presence of non-specific phosphatases. In this study, 5'-nucleotidase was assayed after inhibition of alkaline phosphatase with L-tryptophan. Complete inhibition of alkaline phosphatase was achieved at a concentration of 15 mM L-tryptophan. In our study, a concentration of 20 mM L-tryptophan was routinely used to inhibit alkaline phosphatase.

The change in  $V_{max}$  of both the enzymes without affecting  $K_m$  indicated quantitative alteration of the enzymes during intra-uterine development of the placenta.

The physiological significance of the heat stability of alkaline phosphatase and 5'-nucleotidase in this rapidly developing tissue is still obscure. However, it is assumed that these types of enzymes possess higher half-life in the cells to support rapid growth and proliferation of the tissue (Neale *et al.*, 1965).

The activation of alkaline phosphatase by  $Mg^{2+}$  and inhibition by  $Zn^{2+}$  in human placenta support the findings of Sugiura *et al.* (1977). Contradictory reports are available regarding the effects of EDTA on placental alkaline phosphatase. The present study is in agreement with that of placenta-like alkaline phosphatases from human osteosarcoma cells (Singh *et al.*, 1978), but in contradiction with other reports (Kitchener *et al.*, 1965; Conyers *et al.*, 1966). That  $Mg^{2+}$  is almost ineffective on the activity of placental 5'-nucleotidase is consistent with the findings of Krishnakantha and Maguir (1978) but not with that of Fox and Pamela (1976). Inhibition of 5'-nucleotidase by  $Ni^{2+}$  (in early placenta),  $Zn^{2+}$  and  $Cu^{2+}$  is in conformity with the studies of 5'-nucleotidase from other sources (Ahmed and Reis, 1958).

The inhibition study of placental 5'-nucleotidase by Concanavalin A indicated the glycoprotein nature of the enzyme which is also supported by the studies with the placental enzyme (Krishnakantha and Maguir, 1978) as well as enzymes from other sources (Bhavadasan and Ganguly, 1976; Riordan and Slavik, 1974). Low inhibition of alkaline phosphatase activity by the lectin indicates that the carbohydrate content of the enzyme is very meagre. According to Ahmed and King (1969), human placental alkaline phosphatase contains only traces of carbohydrate, of which the major components are glucosamine and mannose (Hiroshi and Akagi, 1977).

### Acknowledgement

The authors are grateful to Dr S. K. Banerjee, Department of Obstetrics and Gynaecology, SSKM Hospital, Calcutta for permitting them to collect the clinical materials used in this study.

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## Hepatic and extra-hepatic glutathione-S-transferase activity in wild pigeons (*Columba livia*)

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MS received 12 March 1980; revised 30 May 1980

**Abstract.** Glutathione-S-transferase (EC 2.5.1.18) activity was assayed in hepatic and extra-hepatic tissues of pigeons using 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene as substrates. Gluthathione-S-transferase activity towards 1-chloro-2,4-dinitrobenzene in pigeon was in the order: kidney > liver > testes > brain > lung > heart. The enzyme activity with 1-chloro-2,4-dinitrobenzene as substrate was 40-44 times higher in pigeon liver and kidney than that observed with 1,2-dichloro-4-nitrobenzene as substrate.  $K_m$  values of hepatic and renal glutathione transferase with 1-chloro-2,4-dinitrobenzene as substrate were 2.5 and 3 mM respectively. Double reciprocal plots with varying reduced glutathione concentrations resulted in biphasic curves with two  $K_m$  values (liver 0.31 mM and 4 mM; kidney 0.36 mM and 1.3 mM). The enzyme activity was inhibited by oxidized glutathione in a dose-dependent pattern. 3-Methylcholanthrene elicited about 50% induction of hepatic glutathione transferase activity whereas phenobarbital was ineffective.

**Keywords.** Avian glutathione-S-transferase; oxidized glutathione; 3-methylcholanthrene; phenobarbital; inhibition by oxidized glutathione; glutathione-S-transferase.

### Introduction

Glutathione-S-transferases, a family of cytosolic enzymes with overlapping substrate specificities, play an important role in the biotransformation of many xenobiotics. Enzymic reactions involving conjugation of glutathione with a variety of electrophiles have been described (Habig *et al.*, 1974; Hayakawa *et al.*, 1975; Chasseaud, 1976; Mukhtar and Bresnick, 1976a; Jerina and Bend, 1977; Jakoby, 1978). S-conjugate formation by glutathione-S-transferases results in detoxification of a wide variety of electrophiles and is the first step in the syntheses of mercapturic acids (Chasseaud, 1976; Jakoby, 1978). Glutathione-S-transferase activity is widely distributed in mammals (Kaplowitz *et al.*, 1976; Mukhtar and Bend, 1977; Mukhtar *et al.*, 1978; Bend *et al.*, 1979; Cantfort *et al.*, 1979), aquatic organisms (James *et al.*, 1979; Nimimo *et al.*, 1979), insects (Motoyama *et al.*, 1978), earthworm (Stenersen *et al.*, 1979) and in plants (Guddewar and Dauterman, 1979).

Abbreviations used: Chloro-dinitrobenzene, 1-chloro-2, 4-dinitrobenzene; dichlorodinitrobenzene 1,2-dichloro-4-nitrobenzene; GSH, glutathione (reduced); GSSG, glutathione (oxidized); 3MC, 3-methylcholanthrene.

Birds are the first victims of exposure to toxic chemicals in the air and as such it was of interest to study the mechanism of xenobiotic biotransformation in birds. A preliminary report on the presence of glutathione-S-transferase activity in wild birds has appeared (Witt and Snell, 1968). To the best of our knowledge no detailed systematic characterization of glutathione-S-transferase of avian species has been attempted so far. The present paper deals with the occurrence, localization and characterization of glutathione-S-transferase activity in wild pigeon (*Columba livia*).

### Materials and methods

1,2-Dichloro-4-nitrobenzene (dichloronitrobenzene) and 1-chloro-2, 4-dinitrobenzene (chlorodinitrobenzene) were supplied by Eastman Organic Chemicals and the Aldrich Chemical Co., Milwaukee, Wisconsin, USA, respectively, and were recrystallized from ethanol prior to use. Gluthathione reduced (GSH) and glutathione oxidized (GSSG) were purchased from SISCO Research Laboratories, Bombay. 3-Methylcholanthrene was a product of Sigma Chemical Co., St. Louis, Missouri, USA.

### Animals

Male wild pigeons (200-250 g) were obtained with the help of commercial trappers. Albino rats (150-200 g) derived from the animal breeding colony of this Centre and raised on a commercial pellet diet (Hindustan Lever, Bombay) were used.

### Subcellular fractionation:

Pigeons or albino rats were exsanguinated, cut open from abdomen, and the desired tissues were removed, blotted free of blood, washed twice in ice-cold 0.05 M phosphate buffer (pH 7.4), containing 0.15 M KCl. Tissue homogenates (25% w/v) were prepared in buffered KCl using a Potter-Elvehjem glass homogenizer fitted with a teflon pestle. The homogenates were centrifuged at 3000 g for 15 min and the resulting supernatant was recentrifuged at 9000 g for 20 min to recover the postmitochondrial fraction. When needed the mitochondrial fraction was washed twice before use. Cytosolic fraction was obtained by centrifuging the post-mitochondrial supernatant at 105,000 g for 60 min in a Beckman ultracentrifuge. The resulting supernatant was carefully transferred to another tube and was kept at -16°C till the enzyme activity was assayed. Storage upto a week did not decrease the enzyme activity.

### Enzyme assay:

Glutathione transferase activity using chlorodinitrobenzene or dichloronitrobenzene as substrates was assayed spectrophotometrically essentially as described by Habig *et al.* (1971). The cuvettes (final volume of 3.0 ml) contained 0.1 M phosphate buffer (pH 6.5 for assaying chlorodinitrobenzene and pH 7.8 for dichloronitrobenzene activity), 1 mM GSH and 1 mM of either substrate and suitable aliquots (usually 20 µl) of appropriately diluted enzyme from the different sources. Change in absorbance at 340 nm (for chlorodinitrobenzene) and 344 nm (for dichloronitrobenzene) was followed against a blank containing all reactants excepting enzyme protein. Specific activity was expressed as nmol conjugate formed/min/mg protein

using a molar extinction coefficient of 9.6 and 8.5 for chlorodinitrobenzene and dichloronitrobenzene respectively. Protein was determined according to Lowry *et al.*, (1951) using bovine serum albumin as standard.

## Results

Glutathione-S-transferase activity towards chlorodinitrobenzene in kidney and liver cytosols of male pigeons was linear upto 160 µg of protein in the incubation mixture at pH 6.5. In subsequent experiments all the assays were done at this or lower protein concentrations. The effect of varying pH of incubation mixture on hepatic and renal cytosolic glutathione-S-transferase activity with chlorodinitrobenzene as substrate is given in table 1. It was seen that the highest specific activity

**Table 1.** Effect of pH on glutathione-Stransferase activity towards chlorodinitrobenzene as substrate using pigeon liver and kidney cytosols.

pH	nmol conjugate formed/ min/mg of protein	
	Liver	Kidney
5.5	100	140
6.0	251	491
6.5	552	632
7.0	750	843
7.5	1255	1250
8.0	1192	1545

The experiment was repeated three times and almost identical values were obtained. Data of one such experiment is given. All assays were done using phosphate buffer 0.1 M of desired pH value.

was observed at pH 7.5 and 8.0 for liver and kidney glutathione-S-transferase respectively. At higher pH values (7.0 or more) nonenzymatic rate was very high. A compromise was thus made and for all subsequent experiments pH 6.5 was used.

Localization of the enzyme in different sub-cellular fractions of pigeon liver is illustrated by the results summarized in table 2 indicating that almost all the activity is located in the particulate-free cytosol fraction. A relative enrichment of 60% in the specific activity in the cytosol fraction as compared to total homogenate was observed.

The relative specific activities of glutathione-S-transferase in different tissues of pigeon and their comparison with corresponding activities of rat tissues are given in table 3. It could be seen that the activity of enzyme in pigeon tissues was in the order: kidney > liver > testes > brain > lung > heart, whereas in rat a different pattern was observed and the activities were in the order: testes > liver > brain > kidney > heart > lung. Glutathione-S-transferase activity in kidney cytosols of

**Table 2.** Specific activity of glutathione-S-transferase in different subcellular fractions of pigeon liver

Fraction	Specific activity (nmol conjugate formed/ min/mg protein)
Homogenate	248
Nucleus	N.D.
Mitochondria	N.D.
Microsome	N.D.
105,000 g supernatant	391

Experiment was repeated 3 times and almost identical results were obtained. The data of one such experiment is given. Chloronitrobenzene was used as substrate.

N.D = not detectable.

**Table 3.** Glutathione-S-transferase activity with chlorodinitrobenzene as the substrate in pigeon and rat tissue cytosols

Tissue	Specific activity (nmol conjugate formed/ min/mg protein)	
	Pigeon	Rat
Kidney	786	237
Liver	391	1625
Testes	343	1666
Brain	178	379
Lung	123	120
Heart	101	188

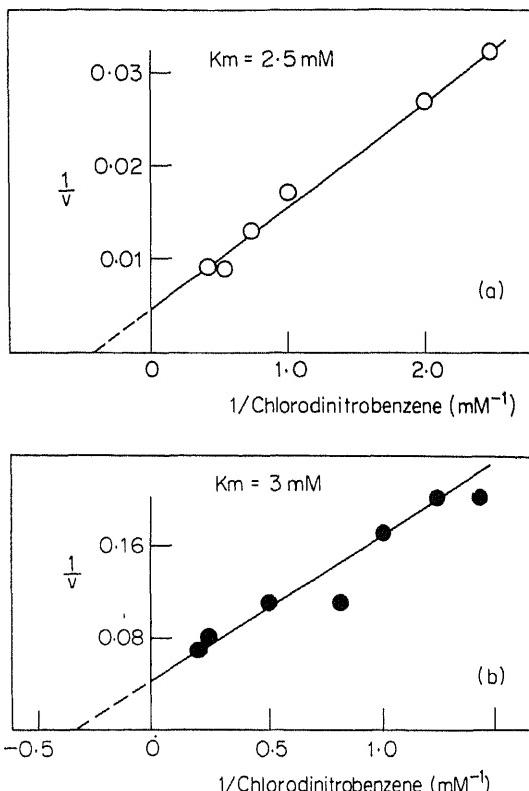
The values given are from a pooled tissue preparation from 4 animals.

pigeon was 3.3 times that of rat kidney, whereas pigeon liver had only 24% of the activity of rat liver. Glutathione-S-transferase activity with chlorodinitrobenzene as substrate in pigeon liver and kidney was 40-44 times higher than that observed with dichloronitrobenzene as substrate (table 4).

The apparent  $K_m$  values for chlorodinitrobenzene were about 2.5 and 3.0 mM for the liver and kidney transferases (figure 1a, b). Lineweaver-Burk plots for glutathione-S-transferase activity with varying glutathione concentrations gave a biphasic curve

**Table 4.** Comparison of glutathione-S-transferase activity in pigeon liver and kidney using chlorodinitrobenzene and dichloronitrobenzene.

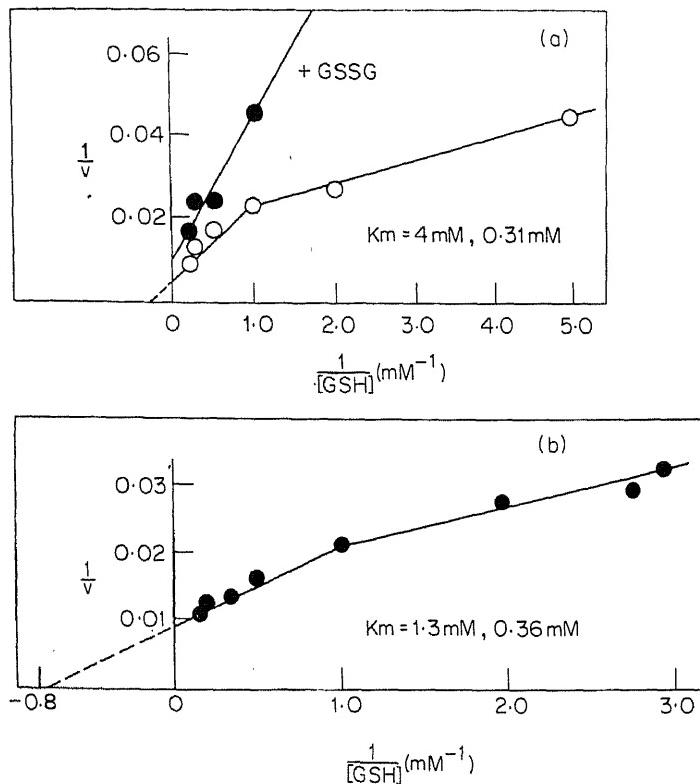
Substrates	Specific activity (nmol conjugate formed/ min/mg protein)	
	Liver	Kidney
Chlorodinitrobenzene	597	800
Dichloronitrobenzene	15	18
Chlorodinitrobenzene/ dichloronitrobenzene	40	44



**Figure 1(a).** Lineweaver-Burk plot showing the effect of varying concentrations of chlorodinitrobenzene on the reaction rate of pigeon liver glutathione transferase. Assays were conducted using liver cytosol as the enzyme source ( $100 \mu\text{g}$  protein). Concentration of the substrate (0.3 to 1 mM) was varied and that of glutathione-reduced (1 mM) was kept constant. Other conditions were as described in the text.

**Figure 1(b).** Lineweaver-Burk plot showing the effect of chlorodinitrobenzene concentration on the reaction rate of pigeon kidney glutathione-S-transferase. Assays were conducted using kidney cytosol as enzyme source ( $100 \mu\text{g}$  protein). Concentration of the substrate (0.5 to 2 mM) was varied and that of GSH (1 mM) was kept fixed. Other conditions were as described in text.

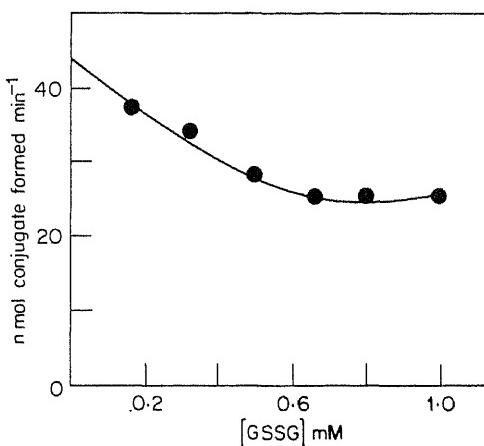
both for liver and kidney enzyme giving  $2K_m$  values; liver 4.0, 0.31 mM and kidney 1.3, 0.36 mM (figure 2a, b). Pigeon liver glutathione-S-transferase activity was inhibited by GSSG in a dose-dependent pattern (figure 3). Figure 2a also indicates that GSSG was a noncompetitive inhibitor of pigeon hepatic glutathione-S-transferase as evident by change brought about in  $V_{max}$  value by GSSG.



**Figure 2(a).** Lineweaver-Burk plot showing the effect of GSH concentration on the rate of the reaction catalyzed by pigeon liver glutathione-S-transferase. Enzyme activity was assayed using liver cytosol as the enzyme source (100 µg protein), in the presence of varying concentrations of GSH (0.2 to 1 mM) and fixed concentrations of chlorodinitrobenzene (1 mM). The velocity in the presence of varying concentrations of GSH and at a fixed concentration of GSSG (1 mM) was determined. GSH, O; GSH+GSSG, ●.

**Figure 2(b).** Lineweaver-Burk plot showing the effect of GSH concentration on the reaction rate of pigeon kidney glutathione transferase. Enzyme activity was assayed using kidney cytosol as the enzyme source (100 µg protein), in the presence of varying concentrations of GSH (0.3 to 1 mM) and fixed concentrations of chlorodinitrobenzene (1 mM).

The effect of phenobarbital or 3-MC administration on hepatic glutathione-S-transferase with chlorodinitrobenzene as substrate is shown by results given in table 5. Phenobarbital at the dose and time period studied did not produce any effect on the levels of hepatic glutathione-S-transferase whereas 3MC stimulated the activity by 65%.



**Figure 3.** Effect of GSSG concentration on the reaction catalyzed by the pigeon liver glutathione-S-transferase. Varying amounts of GSSG (0.1 to 1 mM) were pre-incubated for 10 min at room temperature with the components of the reaction mixture (100 µg enzyme protein). The enzyme activity was inhibited by the addition of chlorodinitrobenzene and was assayed as described in the text.

**Table 5.** Effect of 3-methylcholanthrene and phenobarbital administration to pigeon on hepatic glutathione-S-transferase activity.

Treatment	Specific activity (nmol conjugate formed/ min/mg protein)
Saline	473±147 (4)
Phenobarbital (72 h)	442±129 (4)
Peanut oil	585±163 (4)
3-Methylcholanthrene (48 h)	967±199 <sup>a</sup> (3)

Chlorodinitrobenzene was used as substrate to measure the activity. The data represent mean±S.D. of number of values given in parenthesis. Pigeons were injected intraperitoneally with phenobarbital (40 mg/kg body weight in saline on 3 consecutive days) or with 3-methylcholanthrene (40 mg/kg body weight, in peanut oil for 2 consecutive days). The pigeons were sacrificed 24 h after they had received the last treatment. Control animals received identical volume of saline or peanut oil. All injections or sacrifice were done between 10 and 11 A.M. to avoid any diurnal variation.  
<sup>a</sup>p<0.05.

### Discussion

Avian species are the major targets of attack, by air pollutants. Wild species of birds are equipped with cytochrome P-450 dependent mixed function oxidase system (Runnels and Khan, 1975; Sifri *et al.*, 1975). Earlier studies from this laboratory revealed that tissues of wild pigeon possess considerable activity (as compared to rats) of drug metabolising enzymes and are, therefore, equipped for disposal of xenobiotics (Husain *et al.*, 1979). The present studies demonstrate that, in addition to the mixed function oxidase system, wild pigeons are also equipped with cytosolic glutathione-S-transferases. The presence of significant amounts of glutathione transferase activities in hepatic and extra-hepatic tissues of pigeons is similar to the pattern of enzyme profile in rodent tissues (Bend *et al.*, 1979) suggesting that most target tissues of pigeons are equipped to deal with the toxic effects of circulating electrophiles. In the pattern of localization of glutathione-S-transferase in tissue fractions, the manifestation of a biphasic Lineweaver-Burk plot with GSH and inhibition of enzyme activity by GSSG, the pigeon enzyme shows similarities to the rodent enzyme. Unlike the rodents, pigeons have, however, the highest activity in renal tissue. The enzyme in the kidney glutathione transferase is implicated in the transport of organic anions besides its role in the disposal of xenobiotics as mercapturic acid (Pegg and Hook, 1976). A high enzyme activity in renal tissue of pigeons may also be related to the operation of an overall excretory mechanism different from that of mammals.

Administration of 3-MC to pigeons resulted in the induction of hepatic enzyme activity whereas phenobarbital was ineffective. All the mammalian species investigated so far have shown response to pheno-barbital as inducer of hepatic enzyme activity whereas 3-MC is an inducer in only a few mammalian species. Thus Mukhtar and Bresnick (1976) reported that, in case of rat liver 3MC and phenobarbital are inducers of enzyme activity for most substrates investigated, whereas for mouse liver 3MC was ineffective (Mukhtar and Bresnick, 1976b). Since the molecular mechanism of induction of the enzyme by selective inducers has not been elucidated, it is difficult to ascertain at this stage, the cause of such an unusual induction pattern of the enzyme activity in pigeons.

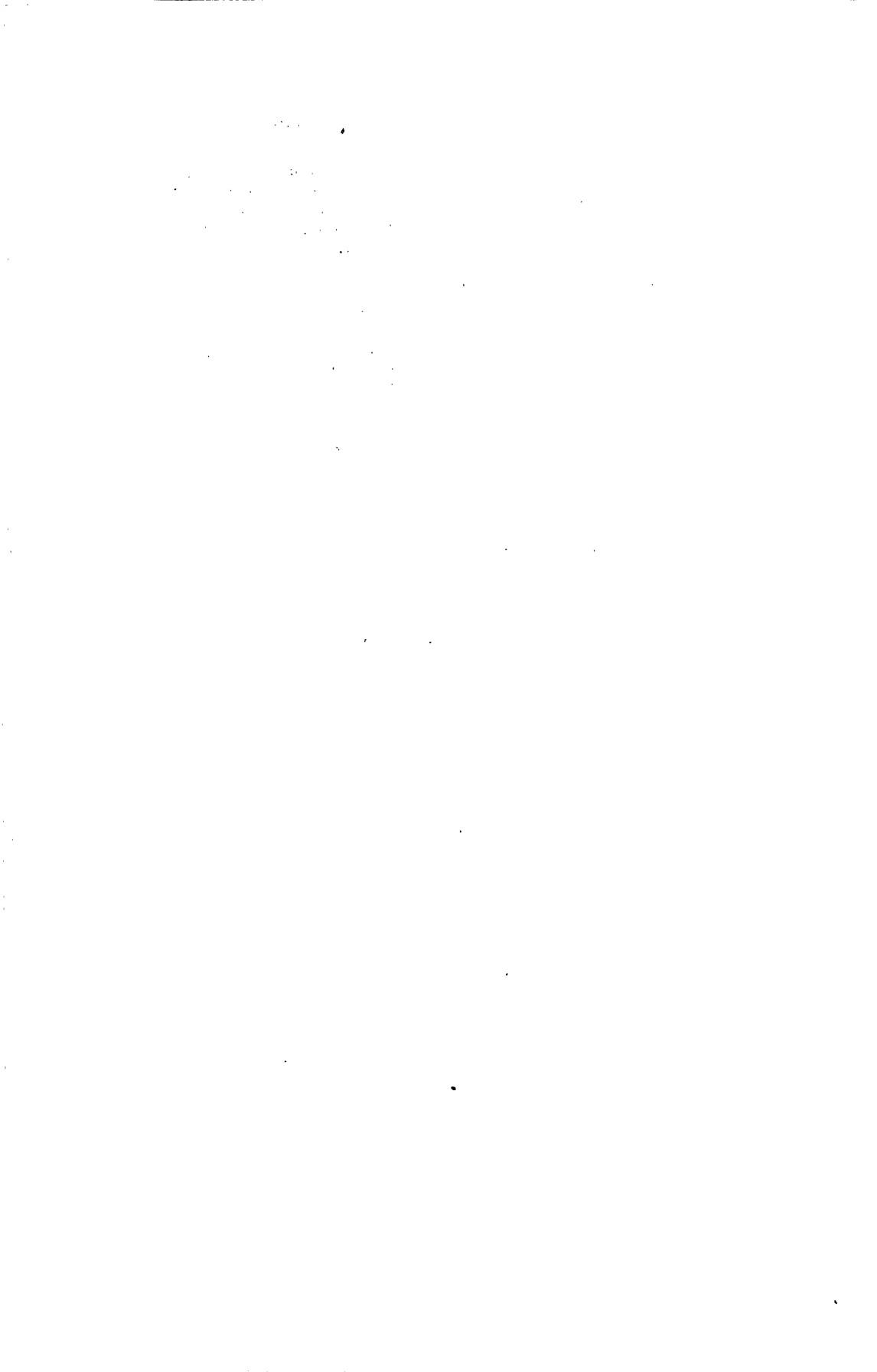
### Acknowledgement

Technical assistance of Mr. N. Singh is acknowledged with appreciation.

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## Galactosyltransferase from buffalo milk: Further characterization

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MS received 26 April 1980; revised 16 July 1980

**Abstract.** Buffalo milk galactosyltransferase is a single poly-peptide of molecular weight 55,000 to 56,000. The enzyme is specific for glucose as an acceptor substrate in the presence of  $\alpha$ -lactalbumin, L-Arabinose, L-xylose, D-ribose and D-fructose did not serve as acceptor substrates even at concentration as high as 0.13 M, while N-acetylglucosamine and ovalbumin served as good acceptors of galactosyl moiety in the absence of  $\alpha$ -lactalbumin. UDP-galacturonic acid did not serve as a donor substrate; on the contrary, it inhibited the reaction. Lactose synthetase reaction was inhibited by D-ribose, L-arabinose and L-xylose, whereas D-fructose did not show any inhibition. Buffalo milk  $\alpha$ -lactalbumin enhanced the synthesis of lactose but inhibited the synthesis of N-acetyllactosamine. Cations like  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Ba}^{2+}$  and  $\text{Co}^{2+}$  could not replace  $\text{Mn}^{2+}$  in the N-acetyllactosamine synthetase reaction. Except  $\text{Co}^{2+}$ , these cations had no effect on this reaction.  $\text{Co}^{2+}$  was found to be a competitive inhibitor of  $\text{Mn}^{2+}$ . The observed inhibition of the reaction by-EDTA also confirmed the absolute requirement of  $\text{Mn}^{2+}$  for the reaction. Lactose synthetase reaction had an optimum pH of 8.5, whereas N-acetyllactosamine synthetase reaction was maximal at pH 8.0.

**Keywords.** Galactosyltransferase; buffalo milk; lactose synthetase.

### Introduction

Galactosyltransferase (EC 2.4.1.38) catalyzes the formation of N-acetyllactosamine from UDP-galactose (UDP-gal) and N-acetylglucosamine (GlcNAc). Besides, the enzyme also synthesizes lactose using glucose as an acceptor of galactosyl moiety in presence of  $\alpha$ -lactalbumin (Brew *et al.*, 1968). Extensive studies have been carried out on galactosyltransferases from bovine (Hill *et al.*, 1968; Morrison and Ebner, 1971a, b, c; Ebner, 1973) and human milk (Andrews, 1970; Khatra *et al.*, 1974). Galactosyltransferases from other sources like human serum (Kim *et al.*, 1972), human cerebrospinal fluid (Ko *et al.*, 1973), rat liver (Fraser and Mookerjee, 1977), chloroplast envelopes of soybean cotyledons (Dalgarn *et al.*, 1979) and *Neurospora crassa* (Forsthoefel and Mishra, 1977) have also been studied. The Purification and some kinetic properties of the buffalo milk enzyme have already been reported (Mahajan, *et al.*, 1979). In this communication, we report further characterization of the buffalo milk galactosyltransferase.

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Abbreviations used: GlcNAc: N-acetylglucosamine, UDP-gal: UDP-galactose, SDS: sodium dodecyl sulphate.

## Materials and methods

### Materials

NADH, phosphoenol pyruvate, pyruvate kinase type I (EC 2.7.1.40), containing 30 units of lactate dehydrogenase (EC 1.1.1.27) activity per mg protein, GlcNAc, bovine serum albumin and UDP-gal were purchased from Sigma Chemical, Co. St. Louis, Missouri, USA. UDP-galacturonic acid was from Calbiochem, Richmond, California, USA. Tris and 2-mercaptoethanol were purchased from E. Merck, Dermstadt, Germany and  $\alpha$ -chymotrypsin was from Nutritional Bio-Chemical Corporation, Cleveland, Ohio, USA. All other reagents used were of analytical grade and were purchased either from BDH or S. Merck, India.

### Methods

Galactosyltransferase was purified from buffalo milk by ammonium sulphate precipitation, hydrophobic chromatography on norleucine-Sepharose and affinity chromatography on  $\alpha$ -lactalbumin-Sepharose. Concurrently  $\alpha$ -lactalbumin was also isolated and purified from buffalo milk (Mahajan *et al.*, 1979).

Galactosyltransferase was assayed spectrophotometrically using Beckman spectrophotometer model 25, by the method of Ebner *et al.*, (1972). When glucose was used as an acceptor substrate,  $\alpha$ -lactalbumin was included in the assay mixture. This reaction has been designated as the 'lactose synthetase' activity of the enzyme. N-acetyllactosamine synthetase activity of the enzyme was assayed using GlcNAc as an acceptor substrate in the absence of  $\alpha$ -lactalbumin. Concentrations of different reagents in the assay mixture (1 ml) were as follows: glycine, 50 mM pH 8.5; MnCl<sub>2</sub>, 5 mM; NADH, 0.083 mM; phosphoenol pyruvate, 0.8 mM; pyruvate kinase, 21.0 units;  $\alpha$ -lactalbumin, 200  $\mu$ g; UDP-gal, 0.23 mM; and either glucose 20 mM or GlcNAc, 20 mM. About 8-10  $\mu$ g of enzyme, obtained after chromatography on  $\alpha$ -lactalbumin-Sepharose column, was used in each assay mixture. One unit of enzyme activity is defined as the amount of enzyme needed for the formation of 1  $\mu$  mol of UDP per min at 25°C.

Polyacrylamide gel electrophoresis of galactosyltransferase was performed using 7.5% gels, prepared according to the method of Davis (1964). Fifty mM potassium phosphate buffer pH 8.0 (Gomori, 1955), was used in the buffer chambers. The gels were stained with either Coomassie Brilliant Blue for protein detection or with periodate-Schiff's base stain for the detection of glycoproteins (Zacharius *et al.*, 1969).

Molecular weight of galactosyltransferase was estimated by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (Weber and Osborn, 1969), as well as by using gel filtration on Sephadex G-100.  $K_{av}$  was calculated as described by Reiland (1971).

The different kinetic properties of the enzyme, namely, substrate specificity, effect of varying concentrations of  $\alpha$ -lactalbumin, effect of various cations and effect of pH were studied using the spectrophotometric assay. The inhibition of lactose synthetase reaction by various sugars as well as the inhibitory effects of UDP-galacturonic acid, EDTA and Co<sup>2+</sup> on the N-acetyllactosamine synthetase reaction were also studied.

## Results

### Molecular weight estimation

Using a calibrated Sephadex G-100 column, the molecular weight of the enzyme was estimated to be 56,000. The data, when replotted in the form of log of molecular weight against  $K_{av}$ , gave the same value (figure 1). SDS-gel electrophoresis under non-denaturing conditions gave a value of 55,000.

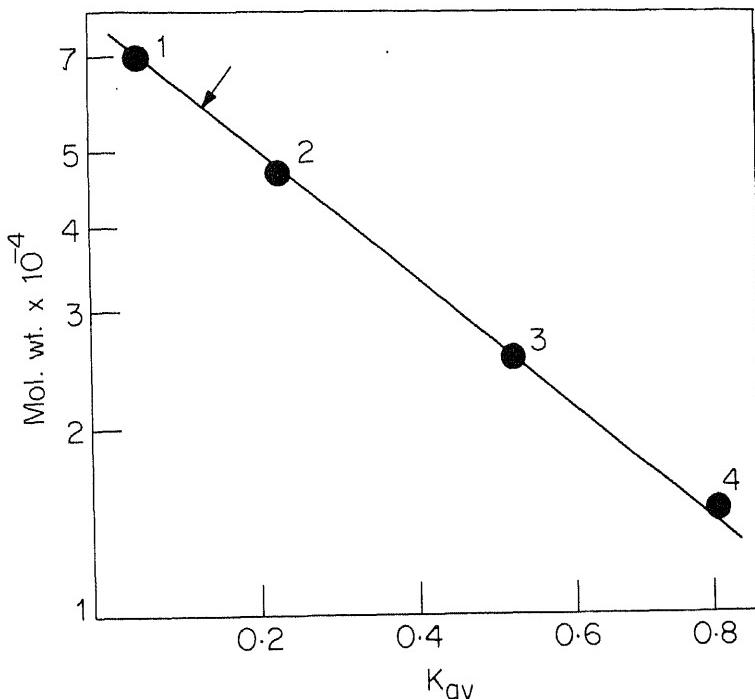


Figure 1. Estimation of molecular weight of buffalo milk galactosyltransferase using Sephadex G-100.

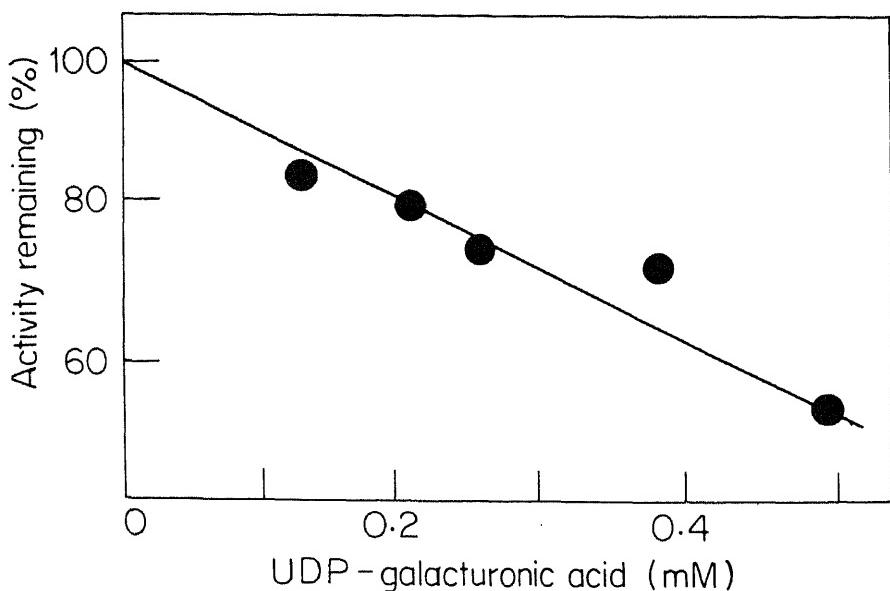
Sephadex G-100 column (0.9×100 cm) was equilibrated and developed with 20 mM Tris-HCl buffer, pH 7.5 containing 0.1 M KCl. The column was calibrated using (i) bovine serum albumin (68,000), (ii) ovalbumin (45,000), (iii)  $\alpha$ -chymotrypsin (24,000) and (iv) bovine  $\alpha$ -lactalbumin (14,500) as marker proteins. The numbers in parentheses indicate molecular weights. Arrow indicates the position of galactosyltransferase.

### Substrate specificity

Buffalo milk galactosyltransferase was specific for glucose as an acceptor of galactosyl moiety in the presence of  $\alpha$ -lactalbumin. L-Arabinose, L-xylose, D-ribose and D-fructose could not replace glucose at concentrations even as high as 130 mM. In the absence of  $\alpha$ -lactalbumin, however, the enzyme was found to be more specific for GlcNAc as an acceptor substrate. Ovalbumin, a glycoprotein, could also accept galactose in the absence of  $\alpha$ -lactalbumin. UDP-galacturonic acid could not replace UDP-gal in the reaction mixture.

### *Inhibition by substrate analogues*

None of the sugars studied inhibited lactose synthetase reaction at lower concentrations. However, at 130 mM concentration, L-arabinose showed 39% inhibition, D-ribose showed 46% inhibition, whereas L-xylose showed only 16% inhibition, and the enzyme D-fructose did not inhibit. UDP-galacturonic acid, an analogue of UDP-gal, inhibited the N-acetyllactosamine synthetase reaction. A maximum of 48% inhibition was obtained at 0.5 mM UDP-galacturonic acid concentration (figure 2).

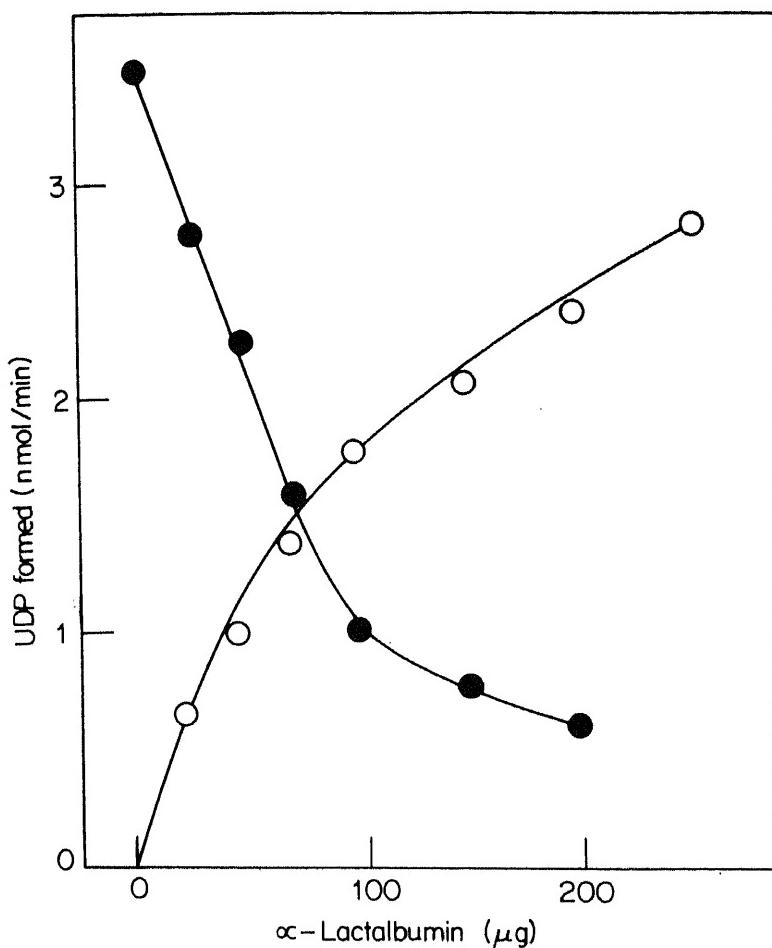


**Figure 2.** Effect of varying concentrations of UDP-galacturonic acid on N-acetyllactosamine synthetase reaction of buffalo milk galactosyltransferase.

Reactions were carried out in the absence of  $\alpha$ -lactalbumin and at fixed concentrations of UDP-gal (0.23 mM). Concentrations of other reagents in the reaction mixture were as described in the text.

### *Effect of $\alpha$ -lactalbumin*

The rate of lactose synthetase reaction increased with increasing concentrations of  $\alpha$ -lactalbumin in the reaction mixture, when the reaction was studied at 20 mM glucose concentration. However, the N-acetyllactosamine synthetase reaction was inhibited by  $\alpha$ -lactalbumin. A maximum of 82% inhibition was obtained at 200  $\mu$ g/ml  $\alpha$ -lactalbumin (figure 3).



**Figure 3.** Effect of buffalo milk  $\alpha$ -lactalbumin on reactions of buffalo milk galactosyltransferase. Lactose synthetase (O) reaction was studied in the presence of different concentrations of buffalo milk  $\alpha$ -lactalbumin with 20 mM glucose; N-acetyllactosamine synthetase (●) reaction was studied with 20 mM GlcNAc. Concentrations of all other reagents in the reactions were as described in the text.

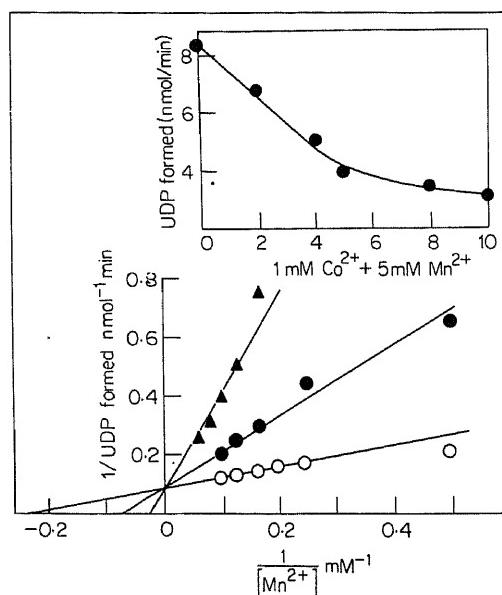
#### *Effect of cations*

Of the various cations studied, none could replace  $Mn^{2+}$  in the N-acetyllactosamine synthetase reaction. A negligible reduction in the rate of reaction was observed when either  $Ca^{2+}$  or  $Mg^{2+}$  was present at 5 mM concentration in the reaction mixture, while  $Cu^{2+}$  or  $Ba^{2+}$  at this concentration did not affect the rate of the reaction. Addition of 5 mM  $Hg^{2+}$  caused precipitation of the reaction mixture. A marked inhibition of the reaction was observed with  $Co^{2+}$  (table 1). Approximately 50% inhibition was observed when the concentrations of  $Co^{2+}$  and  $Mn^{2+}$  were equal in the reaction mixture (figure 4, inset). In another set of experiments, reactions were

**Table 1.** Effect of various cations on the N-acetyllactosamine synthetase reaction of buffalo milk galactosyltransferase.

Cation (5 mM)	nmol UDP formed min <sup>-1</sup>
None	2.96
Ca <sup>2+</sup>	2.80
Mg <sup>2+</sup>	2.80
Ba <sup>2+</sup>	2.90
Co <sup>2+</sup>	1.60
Cu <sup>2+</sup>	2.90
Hg <sup>2+</sup>	caused precipitation

Effect of various cations on the N-acetyllactosamine reaction of buffalo milk enzyme was studied using GlcNAc as the acceptor substrate. Reactions were carried out in the presence of 5 mM Mn<sup>2+</sup>. No reaction was observed in the absence of Mn<sup>2+</sup> and in the presence of 5 mM of individual cation. Concentrations of other reagents in the reaction mixture were as described in the text.

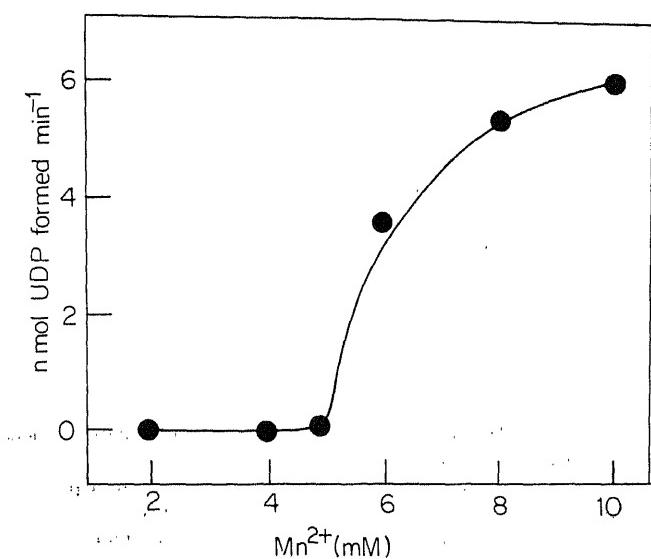


**Figure 4.** Competitive inhibition of Mn<sup>2+</sup> by Co<sup>2+</sup> in the N-acetyllactosamine synthetase reactions of buffalo milk galactosyltransferase. Co<sup>2+</sup>, O; 4 mM Co<sup>2+</sup>, ●; 8 mM Co<sup>2+</sup>, ▲. Inhibitory effect of Co<sup>2+</sup> on the reaction was studied at different fixed concentrations of Co<sup>2+</sup> and at varying concentrations of Mn<sup>2+</sup>. GlcNAc (20 mM) was used as an acceptor substrate in the absence of  $\alpha$ -lactalbumin. Inset shows the effect of varying concentrations of Co<sup>2+</sup> on N-acetyllactosamine synthetase reaction in presence of 5 mM Mn<sup>2+</sup>. Concentrations of all the other reagents in the reaction mixture were as described in the text.

carried out at different fixed concentrations of  $\text{Co}^{2+}$  and at varying concentrations of  $\text{Mn}^{2+}$ . A family of straight lines was obtained in the double reciprocal plot. Upon extrapolation, these lines intersected at a point on Y axis but the slopes of these lines were different (figure 4).

#### *Inhibition by EDTA*

EDTA inhibited N-acetyllactosamine synthetase reaction of the buffalo enzyme. Complete inhibition of the reaction was observed when the concentrations of EDTA and  $\text{Mn}^{2+}$  in the reaction mixture were equal. The inhibitory effect could be reversed by increasing  $\text{Mn}^{2+}$  concentrations in the reaction mixture (figure 5).



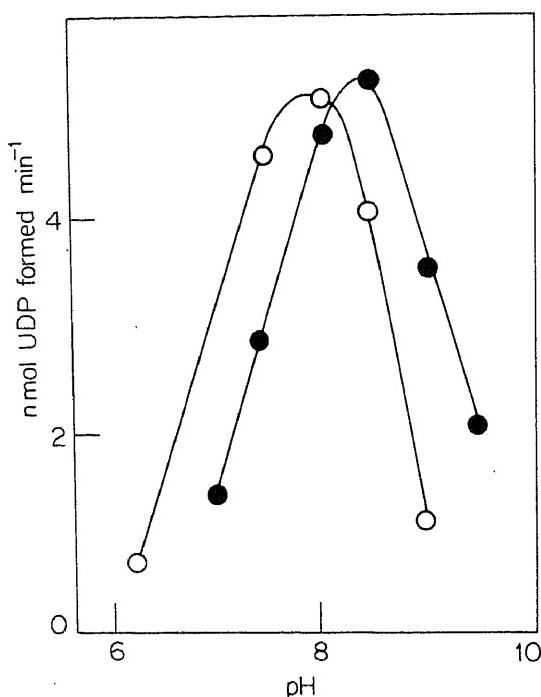
**Figure 5.** Reversible inhibition of N-acetyllactosamine synthetase reaction by EDTA. Effect of EDTA on N-acetyllactosamine synthetase reaction was studied at a fixed concentration of EDTA (5 mM) and at varying concentrations of  $\text{Mn}^{2+}$ . Concentrations of other reagents in the reaction mixture were as described under assay.

#### *Effect of pH*

Effect of varying hydrogen ion concentration on the reactions of galactosyltransferase was studied using 50 mM glycine-KOH buffer in the pH range 6.2 to 9.5 (figure 6). N-Acetyllactosamine synthetase reaction was maximum at pH 8.0, whereas the rate of lactose synthetase reaction was optimal at pH 8.5.

#### **Discussion**

Galactosyltransferase from buffalo milk was made up of a single polypeptide as determined by polyacrylamide gel electrophoresis and SDS-polyacrylamide gel electrophoresis (figure 7). Trayer and Hill (1971) have reported three different



**Figure 6.** Effect of pH on lactose synthetase and N-acetyllactosamine synthetase reactions of buffalo milk galactosyltransferase.

Reactions were carried out using 50 mM glycine-KOH buffer of desired pH. Lactose synthetase reaction (●) was studied using 20 mM glucose and N-acetyllactosamine synthetase reaction (O) in absence of  $\alpha$ -lactalbumin and using 20 mM GlcNAc. Concentrations of other reagents in the reaction mixture were as described in the text.



**Figure 7.** SDS-polyacrylamide gel electrophoresis of buffalo milk galactosyltransferase. Electrophoresis was performed using 10% gels prepared in 0.2 M sodium phosphate buffer, pH 7.2 containing 0.2% SDS and 0.1% 2-mercaptoethanol. Buffer in the electrophoretic chamber was 0.1 M phosphate, pH 7.2, containing 0.1% SDS. About 80  $\mu$ g of SDS treated protein was loaded on each gel and electrophoresis was done at 10 mA per gel for 4 h. Direction of migration was from top to bottom.

forms of bovine enzyme with molecular weights 54,000, 48,000 and 42,000. However, Magee *et al.* (1974) isolated only two active forms of bovine enzyme with molecular weights 55,000-59,000 and 42,000-44,000. The higher molecular weight form of the enzyme is believed to undergo proteolysis (Magee *et al.*, 1976) giving rise to lower molecular weight forms. The enzyme in colostrum, however remained in the higher molecular weight form only, due to the presence of protease inhibitors in the colostrum (Powell and Brew, 1974). Human milk enzyme also exhibited heterogeneity (Prieels *et al.*, 1975). Our studies with the limited number of samples surveyed from buffalo milk did not show any heterogeneity of the enzyme. Its molecular weight as estimated by gel exclusion chromatography was 56,000, while a value of 55,000 was obtained by SDS-gel electrophoresis. The red coloured protein bands observed on staining the polyacrylamide gels with periodate-Schiff's base (Zacharius *et al.*, 1969) suggested that the buffalo milk enzyme was a glycoprotein.

The enzyme was specific for UDP-gal as the donor substrate and was similar to the galactosyltransferases from bovine milk (Ebner *et al.*, 1972), rat serum (Fraser and Mookerjea, 1977), reproductive organs of rodent and human males (Tadolini *et al.*, 1977) and chick embryo (Risteli, 1978). In the present studies, we found that UDP-galacturonic acid could not replace UDP-gal in the reaction, indicating the requirement of an essential  $-\text{CH}_2\text{OH}$  group at the C-6 position of galactosyl moiety. On the contrary, UDP-galacturonic acid inhibited the N-acetyllactosamine synthetase reaction. Similar observations were reported for the enzyme from chick embryo (Risteli, 1978). Recently, a model for the UDP-gal binding site of the enzyme has been proposed (Andree and Berliner, 1978; Berliner and Andree, 1978). The binding site near the C-4 position has been proposed to play a crucial role in determining the substrate specificity. The buffalo enzyme is highly specific for glucose as an acceptor of galactose in the presence of  $\alpha$ -lactalbumin. L-xylose, L-arabinose, D-ribose and D-fructose did not serve as acceptor substrates even in the presence of  $\alpha$ -lactalbumin and at concentrations upto 130 mM. However, the enzyme was inhibited by L-arabinose, L-xylose and D-ribose at concentrations above 30 mM. L-Arabinose, L-xylose, D-ribose and UDP-galacturonic acid did not inhibit the actions of pyruvate kinase and lactate dehydrogenase, the enzymes used in coupled assay system used to estimate galactosyltransferase. Our results are in good agreement with those reported for the bovine enzyme (Morrison and Ebner, 1971b). In the absence of  $\alpha$ -lactalbumin, the enzyme transferred galactose to GlcNAc very efficiently. Ovalbumin was also a good acceptor of galactose in the absence of  $\alpha$ -lactalbumin. Thus, the buffalo milk enzyme was very similar to the bovine milk enzyme (Schanbacher and Ebner, 1970).

The modifier role of buffalo milk  $\alpha$ -lactalbumin was studied using buffalo milk galactosyltransferase. Lactose synthetase activity of the enzyme, when studied at millimolar concentrations of glucose, was enhanced by  $\alpha$ -lactalbumin. However, the rate of N-acetyllactosamine synthesis decreased with increasing concentrations of  $\alpha$ -lactalbumin. Similar observations were reported for the bovine (Ebner, 1970) and human enzymes (Andrews, 1970). Galactosyltransferases from sources other than milk exhibited lactose synthetase activity in the presence of exogenously added  $\alpha$ -lactalbumin. The exception being the cancer-associated isoenzyme of galactosyltransferase which was insensitive to the presence of  $\alpha$ -lactalbumin (Podolsky and Weiser, 1979), and might be of some biological significance.

Galactosyltransferases in general require  $Mn^{2+}$  for their catalytic activity, but the colostrum enzyme was activated by  $Zn^{2+}$ ,  $Cd^{2+}$ ,  $Co^{2+}$ ,  $Fe^{2+}$  or  $Ca^{2+}$  (Powe and Brew, 1976). These workers showed that this enzyme had two metal binding sites, one of them was specific for  $Mn^{2+}$ . In the present studies, complete inhibition of the reaction was observed at EDTA concentrations equal and above the concentration of  $Mn^{2+}$  in the reaction mixture. This inhibitory effect could be reversed by increasing the concentrations of  $Mn^{2+}$  above the total concentration of EDTA in the reaction mixture. This confirms our earlier observations that the buffalo milk enzyme is absolutely dependent on  $Mn^{2+}$  for its activity (Mahajan et al., 1979).

We also observed that none of the metal ions studied could replace  $Mn^{2+}$  in the reactions catalyzed by the enzyme. Morrison and Ebner (1971a) have reported similar observations for the purified bovine enzyme. Although  $Mg^{2+}$  and  $Ca^{2+}$  were reported to replace  $Mn^{2+}$  for the crude bovine enzyme (Ebner et al., 1972) the divalent metal ions like  $Ca^{2+}$ ,  $Mg^{2+}$  and  $Cu^{2+}$  had no effect at all on the rate of the N-acetyllactosamine synthetase reaction of the purified buffalo enzyme. A heavy metal ion, like  $Hg^{2+}$  caused precipitation of the reaction mixture. Only  $Co^{2+}$  showed a significant inhibition.  $Mg^{2+}$ ,  $Co^{2+}$  and  $Mn^{2+}$ , which can form both octahedral and tetrahedral complexes, are activators of pyruvate kinase (Mildvay and Cohn, 1965) while these and other metal ions have no effect on lactate dehydrogenase (Hayaishi, 1966).

A detailed kinetic analysis was carried out to understand the inhibitory effect of  $Co^{2+}$  on the N-acetyllactosamine synthetase reaction. From the typical kinetic pattern obtained, we conclude that  $Co^{2+}$  acts as a competitive inhibitor of  $Mn^{2+}$  consistent with the observations of Risteli (1978) with the chick embryo enzyme. This conclusion is further supported by the fact that  $Co^{2+}$  and  $Mn^{2+}$  have very close ionic radii and have the same charge (Lange, 1964).

Buffalo milk enzyme showed a broader pH profile. The lactose synthetase activity was maximum at pH 8.5, whereas the N-acetyllactosamine synthetase reaction showed an optimum pH of 8.0. Fitzgerald et al., (1970) have reported similar values for the bovine enzyme.

In conclusion it can be said that though galactosyltransferases from cow and buffalo milk differ in the number of their molecular forms and their molecular weights, most of their kinetic properties are similar.

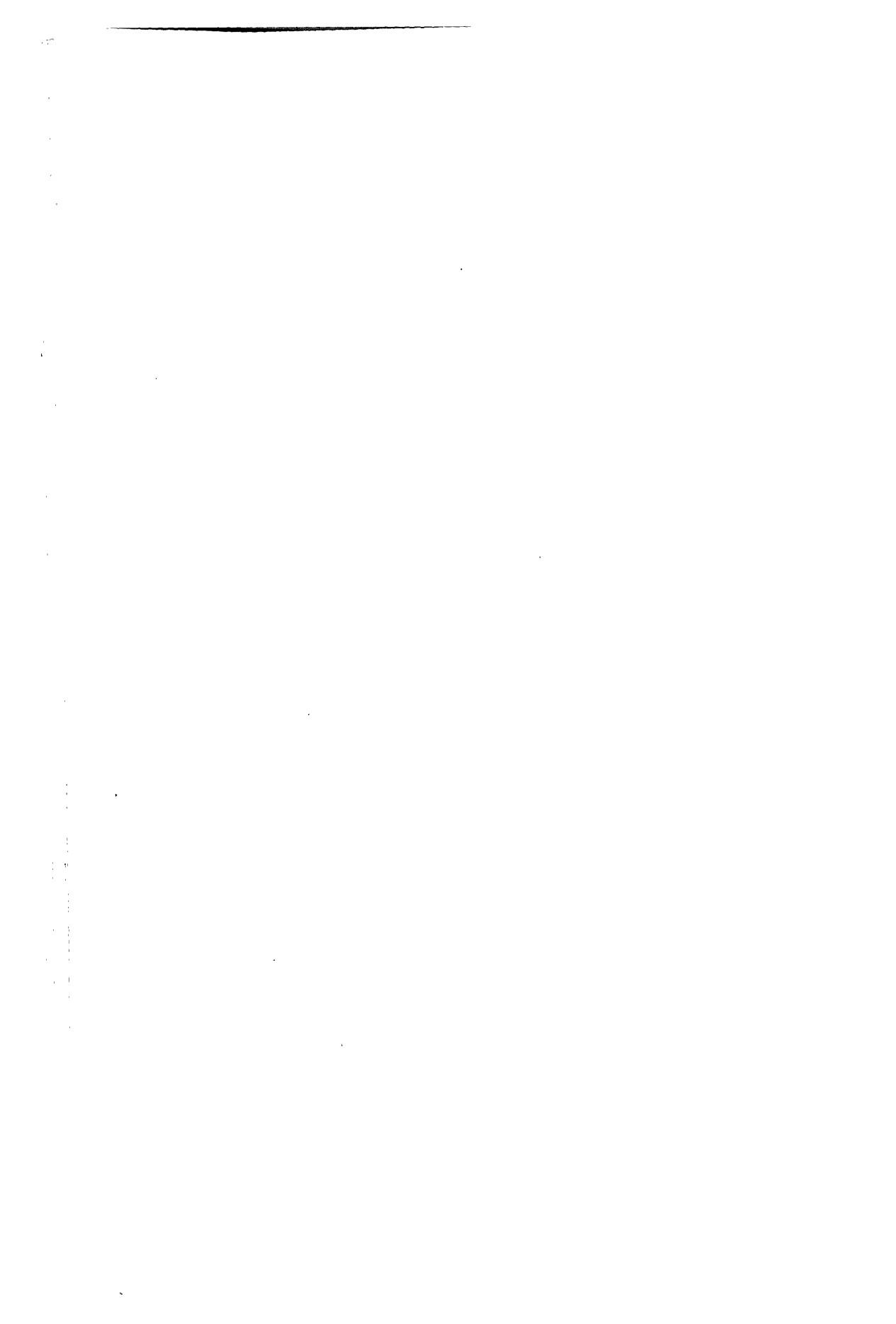
### Acknowledgements

We wish to thank Professor John Barnabas, for his critical suggestions and Mr. Sojar for his assistance. This work was supported by a research grant from Indian Council of Agricultural Research, New Delhi.

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## **Hexokinase isoenzymes in diabetes**

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MS received 10 January 1980; revised 5 June 1980

**Abstract.** Hexokinase is present in the tissues in four isoenzymic forms. Cerebral tissue contains predominantly Type I hexokinase which is believed to be insulin-insensitive. In cerebral tissue about 60 to 70% of the hexokinase is bound to the particulate fraction. The changes in the distribution of hexokinase Type I and Type II together with the bound and free hexokinase have been studied in control, diabetic and diabetic animals treated with insulin. The results indicate that the presence of insulin is essential for the normal binding of the hexokinase to the particulate fraction. In heart tissue, Type II hexokinase bound to the pellet shows a significant decrease in diabetes, which is reversed on insulin administration.

**Keywords.** Hexokinase isoenzymes; alloxan diabetes; insulin; cytosol; total particulate fraction.

### **Introduction**

Hexokinase (EC 2.7.1.1) exists in tissues in four isoenzymic forms (Grossbard and Schimke, 1960; Katzen and Schimke, 1965; Katzen *et al.*, 1975). Type II hexokinase which is known to be insulin-sensitive is found predominantly in adipose tissue and mammary gland (McLean *et al.*, 1967; Katzen, 1967; Walters and McLean, 1968). Cerebral tissues contain predominantly Type I hexokinase (Wilson, 1968, 1972). Recently, we have reported the distribution of hexokinase in brain and demonstrated the presence of about 45% Type II isoenzyme in brain (Ali and Baquer, 1980). A substantial proportion of cerebral hexokinase is bound to sub-cellular particles (Wilson, 1968; Thompson and Bachelard, 1977; Ali and Baquer, 1980).

In the present work an attempt has been made to study the distribution of hexokinase isoenzymes in the brain and heart of control and diabetic rats. Administration of insulin seems to reverse the diabetic effects indicating that the presence of insulin is essential for the stability and binding of hexokinase.

### **Materials and methods**

#### *Animals*

Adult female rats of Holtzman strain weighing between 175-200 g maintained on a standard pelleted diet in our Department were used for the experiments.

#### *Alloxan treatment*

A group of rats was starved for 48 h and each rat received a single subcutaneous injection of alloxan monohydrate prepared freshly in acetate buffer, pH 4.5

20 mg/100 g body wt). Protamine zinc insulin injections (1 unit/rat) were given intra-peritoneally for five days after the alloxan injection. This treatment prevents the high mortality rate of the animals caused by alloxan toxicity. The animals were used three weeks after insulin withdrawal. Rats were provided food and water *ad libitum*.

Most of the chemicals and purified enzymes were from Sigma Chemical Company, St. Louis, Missouri, USA, unless otherwise mentioned.

#### *Tissue extracts*

Rats were sacrificed by cervical dislocation and tissues were excised immediately, weighed and homogenized as described earlier (Baquer *et al.*, 1976). The homogenates were dialyzed for 1 h in the cold against the homogenizing buffer (sucrose 0.25M, triethanolamine, 20 mM; dithiothreitol, 0.1 mM, pH 7.4) to remove low molecular weight compounds like glucose. The extracts were centrifuged at 15,000 g for 40 min, and pellets were suspended in the homogenizing buffer and were referred to as the total particulate fraction in the text. To liberate the latent and bound enzymes, the total particulate fraction was treated in the cold for 30-60 min with Triton X-100 (final concentration 0.5%). Protein was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

#### *Determination of blood sugar*

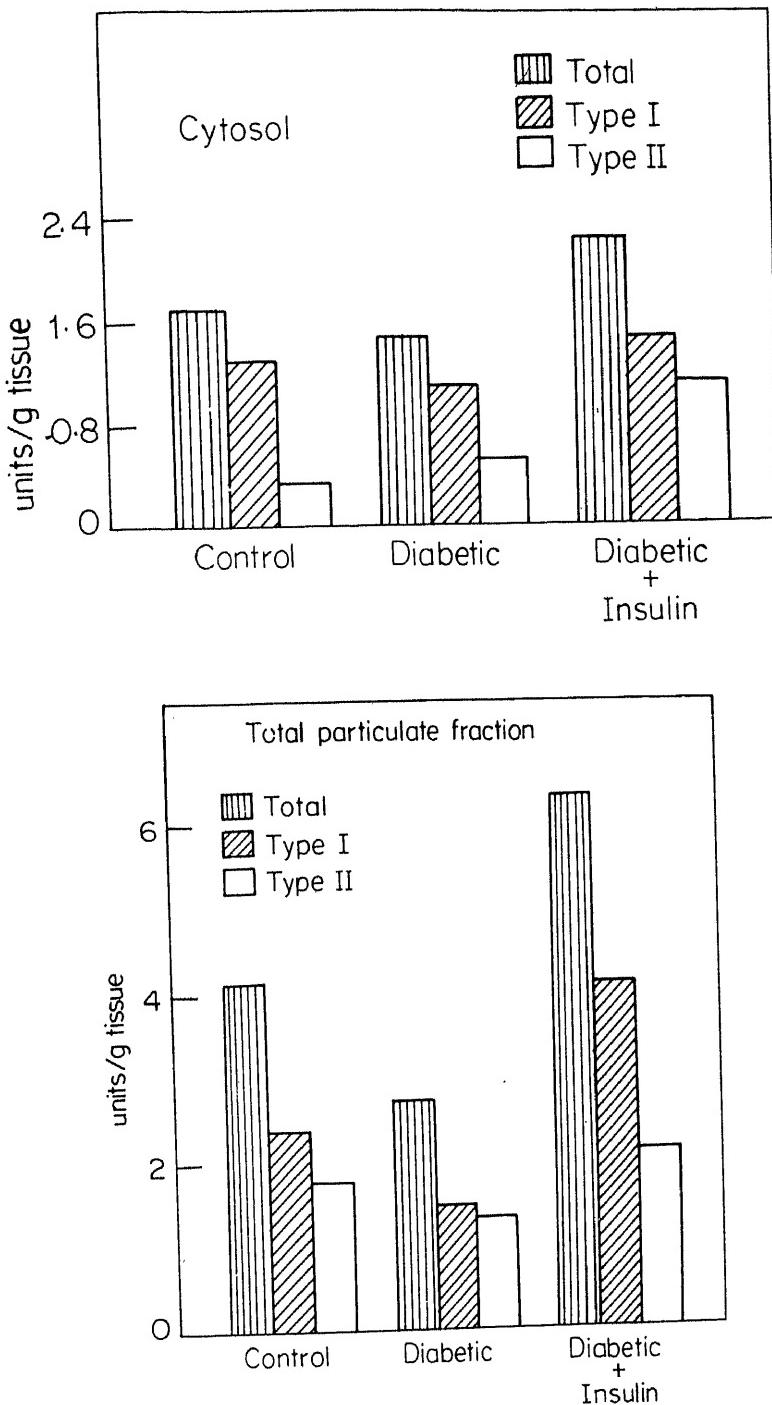
Blood (50 µl) was drawn by heart puncture into 0.2 ml of distilled water. The samples were frozen overnight and thawed the next day. The samples were centrifuged and the supernatant used for the determination of blood glucose by the hexokinase reaction (Bergmeyer *et al.*, 1974).

#### *Enzyme assay*

Hexokinase was estimated essentially according to the method of Sharma *et al.* (1963), as modified by Gumaa and McLean (1972). For the estimation of Type II activity, a portion of each fraction was heated at 45°C for 1 h (Katzen and Schimke, 1965). The reaction mixture contained the following components: Tris-HCl buffer, 20 mM; (pH 7.4); MgCl<sub>2</sub>, 8 mM (pH 7.0); NADP, 0.4 mM, ATP/Mg<sup>2+</sup>, 8 mM/2 mM (pH 7.2); glucose, 5 mM and one unit of purified glucose-6-phosphate dehydrogenase. One unit of activity of hexokinase was defined as the amount required to form one µ mol of NADPH per min at 25°C.

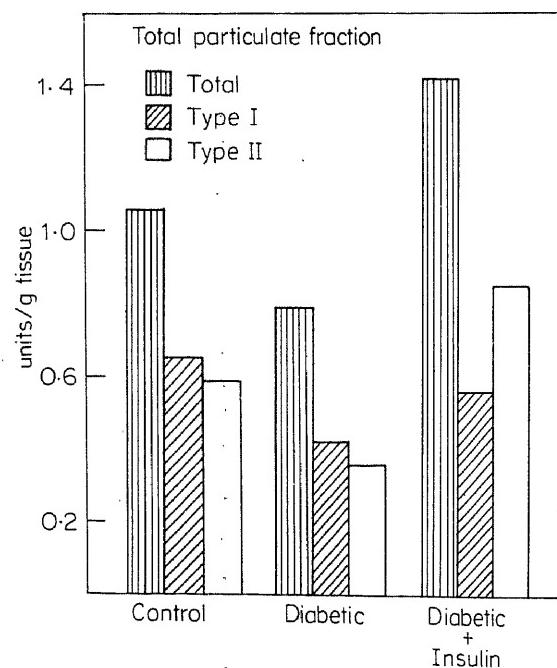
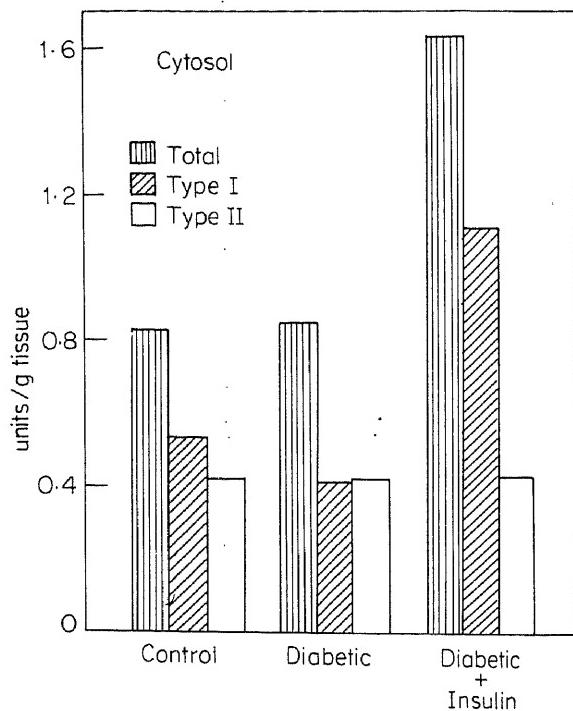
#### **Results**

The results of the present experiment on the distribution of the isoenzymes of hexokinase are presented in figures 1 to 4. Figures 1 and 2 show the pattern in cerebral hemispheres. As can be seen, there was a significant decrease in the amount of Type I bound to the pellet which was increased to values larger than of the controls on the administration of insulin. There was also a marginal (20%) change in the Type II bound to the total particulate fraction, which again increases to more than the control value on insulin administration similar to the pattern observed with Type I. The soluble hexokinase, however, was not altered markedly only the administration of insulin to diabetic animals activated the enzymes to some extent.



**Figures 1 and 2.** Distribution of hexokinase isoenzymes in cerebral hemisphere.

The distribution pattern of heart isoenzymes is shown in Figures 3 and 4. Unlike the hexokinase from cerebral tissue, the total particulate fraction Type I did not



**Figures 3 and 4.** Distribution of hexokinase isoenzymes in heart.

alter significantly, but the Type II bound to the total particulate fraction was decreased in diabetes and the effect was reversed by the administration of insulin. The soluble, total and Type I also increased in activity, similar to the changes in the brain.

The distribution pattern of the hexokinase between the soluble and the total particulate fraction in brain is shown in table 1. A significant decrease in the

**Table 1.** Distribution of hexokinase in the soluble and particulate fractions of brain tissues of the rat

Condition	Total hexokinase	Soluble	Total particulate fraction
(units/g tissue)			
Control	5.8±0.94	1.69±0.26	4.16±0.71
Diabetic	4.59±0.44 (NS)	1.46±0.13 (NS)	2.73±0.29 (NS)
Diabetic + insulin	8.59±0.70 <sup>a</sup>	2.25±0.39 (NS)	6.34±0.33 <sup>a</sup>

Each value is a mean ±SEM of at least four determinations.

<sup>a</sup> : p < 0.05

NS — not significant

enzymes bound to the total particulate fraction occurred with alloxan diabetes. Administration of insulin increased the bound hexokinase accompanied by the activation of the soluble hexokinase. Similar results were found in heart hexokinase as shown in table 2, except that the inactivation of bound hexokinase in diabetes was not as significant as that found in the case of brain.

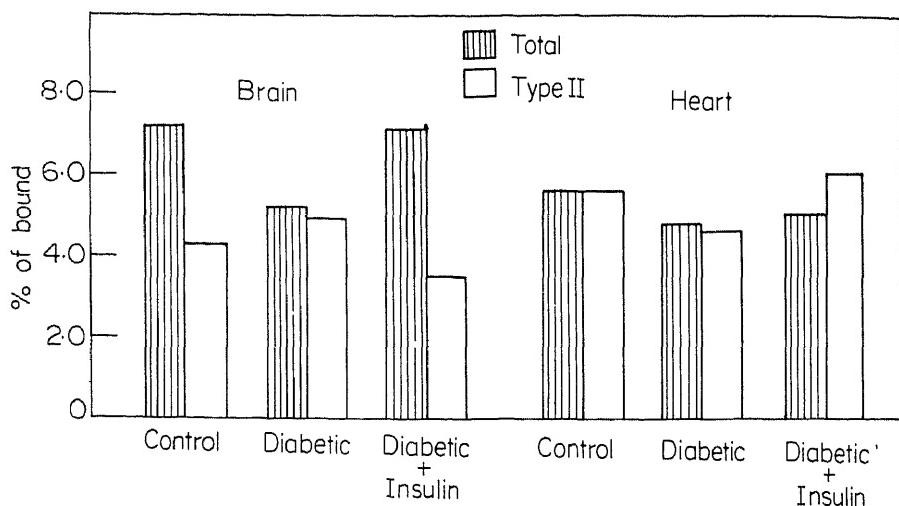
**Table 2.** Distribution of heart hexokinase in the soluble and particulate fractions

Condition	Total hexokinase	Soluble fraction	Total particulate fraction
(units/g tissue)			
Control	1.89±0.10	0.83±0.18	1.06±0.14
Diabetic	1.64±0.17 (NS)	0.85±0.10 (NS)	0.79±0.13 (NS)
Diabetic + insulin	2.89±0.45 (NS)	1.63±0.09 <sup>a</sup>	1.42±0.23 (NS)

Each value is a mean ±SEB of atleast four determinations.

<sup>a</sup> p=0.02.

The percentage of hexokinase bound to the total particulate fraction and the changes with diabetic conditions and insulin administration are shown in figure 5.



**Figure 5.** Percentage of hexokinase bound to total particulate fraction.

The total amount of hexokinase bound to the total particulate fraction in control brain and heart tissues of control animals were 72% and 56% respectively. Induction of diabetes reduced the amount bound to 52% and 48% in the brain and heart. The administration of insulin to diabetic animals restored the reduced levels of brain hexokinase to control values. The percentage of Type I and Type II bound are also shown in figure 5. The weight of the rats, protein values together with blood glucose values are presented in table 3.

**Table 3.** Weight of rats, protein and glucose content of blood, brain and heart tissues of control, diabetic and insulin-treated rats

Treatment	Weight	Blood glucose (mg)	Protein content (mg/g)			
			Brain		Heart	
			Soluble	Pellet	Soluble	Pellet
Control	201±17	77.4±9.9	58±5.0	65.2±5.4	72.0±8.6	57.9±4.8
Diabetic	164±9	270±35	64.0±3.5	66.9±3.1	70.9±3.7	62.3±3.9
Diabetic + insulin	125±11	168±11.5	42.4±4.1	55.0±5.3	51.4±5.6	61.2±7.9

Each value is a mean ±SEM of at least four determinations.

## Discussion

Katzen and Schimke (1965) and Katzen (1966) from a survey of the multiple forms of hexokinase in different normal tissues and from changes found in starved or alloxan diabetic rats for the first time suggested that there might be a correlation between hexokinase Type II and the action of insulin. Several workers (Moore *et al.*, 1964; Katzen, 1966; McLean *et al.*, 1966) had previously demonstrated that the activity of Type II enzyme in adipose tissue decreased in starvation and diabetes, conditions characterized by low blood insulin levels. Katzen (1967) later proposed that the amount of hexokinase Type I relative to that of Type II might be an important factor in determining insulin sensitivity. A relative deficiency in Type I appeared to be correlated with insulin requirement.

Diabetes induced by alloxan is known to cause changes in cerebral metabolites (Thurston *et al.*, 1975). The most significant changes were the levels of glucose-6-phosphate and phosphocreatine. Wilson (1968) had earlier demonstrated the solubilization of brain hexokinase by glucose-6-phosphate. Taking into consideration the above two observations, it was demonstrated that induction of diabetes reduced the amount of hexokinase bound to the total particulate fraction, in both brain and heart (tables 1 and 2). There was, however, no concomitant increase in the soluble enzyme in both the tissues. The increased content of glucose-6-phosphate in cerebral tissues of diabetic animals might be one of the factors responsible for releasing the enzyme from the particulate fraction. It is also probable that the lack of insulin (due to the diabetic state) might cause the inactivation of the hexokinase in the soluble fraction. On insulin administration, the bound enzyme increased in both heart and brain and an increase in activity of the soluble enzyme was also observed. The relative percentage of Type I and Type II bound to the total particulate fraction in brain and heart can be seen clearly in figure 5. Type I isoenzyme was also modified in the diabetic state in both the tissues, the changes in Type II isoenzyme of insulin-sensitive tissues, in a diabetic state is already known and was discussed earlier (Moore *et al.*, 1964; Katzen, 1966; McLean *et al.*, 1966).

Insulin may be of importance both in the binding of hexokinase to cell membranes, in particular to mitochondria and in the stability of hexokinase Type II (Walters and McLean, 1968). The observation reported here substantiates the above view that insulin is an important factor for the normal physiological functioning of the tissues, like the brain and heart which are responsive to the *in vitro* addition of the hormone.

The ability of insulin to increase the *in vitro* content of hexokinase in epididymal adipose tissue has been demonstrated by Hansen *et al.* (1970). Insulin by itself was found to be ineffective but in the presence of an energy source like glucose, it caused a significant stimulation. The increased activity of hexokinase in the diabetic state with insulin administration, as shown in the present results (table 1) may be due to the presence of insulin in a condition with high circulating glucose levels.

Further work is in progress on the activity of other enzymes and metabolites controlling the process of brain glycolysis in diabetes.

## Acknowledgements

The financial assistance of the Council of Scientific and Industrial Research, New Delhi in the form of fellowships to FA and ASNM is gratefully acknowledged.

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## **Comparison of the conformation and stability of the native dimeric, monomeric, tetrameric and the desensitized forms of the nucleotide pyrophosphatase from mung bean (*Phaseolus aureus*) seedlings.**

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MS received 26 May 1980; revised 18 August 1980

**Abstract.** A homogenous and crystalline form of nucleotide pyrophosphatase (EC 3.6.1.9) from *Phaseolus aureus* (mung bean) seedlings was used for the study of the regulation of enzyme activity by adenine nucleotides. The native dimeric form of the enzyme had a helical content of about 65% which was reduced to almost zero values by the addition of AMP. In addition to this change in the helical content, AMP converted the native dimer to a tetramer. Desensitization of AMP regulation, without an alteration of the molecular weight, was achieved either by reversible denaturation with 6 M urea or by passage through a column of Blue Sepharose but addition of *p*-hydroxymercuribenzoate desensitized the enzyme by dissociating the native dimer to a monomer. The changes in the quaternary structure and conformation of the enzyme consequent to AMP interaction or desensitization were monitored by measuring the helical content, EDTA inactivation and Zn<sup>2+</sup> reactivation, stability towards heat denaturation, profiles of urea denaturation and susceptibility towards proteolytic digestion. Based on these results and our earlier work on this enzyme, we propose a model for the regulation of the mung bean nucleotide pyrophosphatase by association-dissociation and conformational changes. The model emphasizes that multiple mechanisms are operative in the desensitization of regulatory proteins.

**Keywords.** *Phaseolus aureus*; nucleotide pyrophosphatase; regulation; desensitization; conformational changes; circular dichroism; optical rotatory dispersion.

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Abbreviations used: Ultraviolet, uv; circular dichroism, CD; optical rotatory dispersion, ORD; sodium dodecyl sulphate, SDS.

## Introduction

There have been several examples of allosteric enzymes that are susceptible to desensitization of their regulatory responses by certain reagents (Carney *et al.*, 1978; Clark and Yielding, 1971; Gerhart and Pardee, 1962; Graves and Wang, 1972; Mangiarotti and Pontremoli, 1963; Wang and Tu, 1969; Weitzman, 1966). The study of the desensitized enzymes, which behave with markedly altered kinetic and physical properties, has been useful in understanding the details of the allosteric regulation of the enzyme activity. Nucleotide pyrophosphatase (dinucleotide nucleotide-hydrolase, EC 3.6.1.9) catalyzes the hydrolysis of dinucleotides at the pyrophosphate bond to yield the corresponding mononucleotides and AMP. It was earlier reported from this laboratory that the enzyme from mung bean seedlings was a dimer ( $M_r$  65,000) and was converted to a tetramer on the addition of AMP. The native dimer was dissociated into a monomer by *p*-hydroxymercuribenzoate. The monomer, dimer and tetramer were all enzymatically active. The dimer was desensitized by treatment with *p*-hydroxymercuribenzoate or by reversible denaturation with urea (Ravindranath and Appaji Rao, 1969; Balakrishnan *et al.*, 1975, 1977). We have recently reported a method for the isolation of a dimeric form of the enzyme which was desensitized to interactions with AMP. The desensitized enzyme had enhanced catalytic activity and increased temperature optimum (Reddy *et al.*, 1979). It was, therefore, of interest to examine the conformation and stability of the desensitized enzyme and compare it with the native dimeric and tetrameric forms of the enzyme. The results of such a study are presented in this paper along with a model to explain the regulation of mung bean nucleotide pyrophosphatase by AMP.

## Materials and methods

### Materials

FAD, AMP, *p*-hydroxymercuribenzoate,  $\alpha$ -chymotrypsin, EDTA, urea, sodium dodecyl sulphate (SDS) and bovine serum albumin were from Sigma Chemical Company, St. Louis, Missouri, USA. Trypsin (diphenyl carbamyl chloride treated) was from Serva Feinbiochemica, Heidelberg, Germany. Urea was recrystallized twice before use. Mung bean seeds were purchased from the local market.

### Methods

The native enzyme, the monomer, the tetramer and the desensitized enzymes were prepared as reported earlier (Ravindranath and Appaji Rao, 1968; Balakrishnan *et al.*, 1974, 1975, 1977; Reddy *et al.*, 1979). The monomeric, dimeric and tetrameric forms of the enzyme were assayed using 0.5 mM FAD and the desensitized enzyme using 1 mM FAD as the substrate (Ravindranath and Appaji Rao, 1968; Reddy *et al.*, 1979). Protein was estimated by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as the standard.

Circular dichroism (CD) and optical rotatory dispersion (ORD) spectra were recorded using a Jasco J-20 automatic recording spectropolarimeter at an appropriate chart speed and time constant to obtain maximal resolution of the spectrum. Calibration of the CD and ORD scales were done using *d*-10-camphor sulphonic acid and

sucrose, respectively (Adler *et al.*, 1973). All measurements were made at 25°C. Quartz cells with a light path of 5 mm were used. The CD and ORD spectra are reported in terms of mean residue ellipticities [ $\theta$ ] and mean residue rotation [ $m$ ] respectively in deg. cm<sup>2</sup>. dmol<sup>-1</sup>, taking a value of 115 for the mean residue weight of the enzyme.

The helical content was calculated from the CD spectra by the method of Greenfield and Fasman (1969) employing the following equation:

$$\% \text{ Helix} = \frac{[\theta]_{208} - 4000}{33000 - 4000} \times 100$$

The helical content was also determined from the ellipticity value at 222 nm and from the ORD data using the following equations (Chen and Yang, 1971):

$$[\theta]_{222} = -30,300 f_H - 2340$$

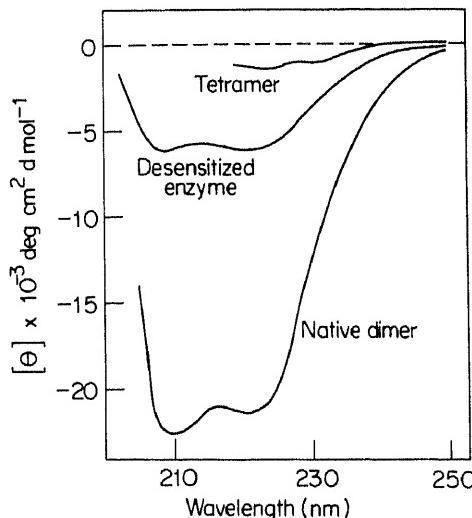
$$[m]_{233} = -12,700 f_H - 2520$$

Where  $f_H$  is the fractional helical content. The reference values of  $[\theta]_{208}$  and  $[m]_{233}$  for fully helical and random coil forms of poly L-lysine were used (Greenfield *et al.*, 1967).

## Results

### *Effect of AMP*

The far-ultraviolet (U.V.) CD spectrum (figure 1) of the native dimer showed two troughs at 208 and 222 nm with mean residue ellipticities of -22,200 and -21,300 deg. cm<sup>2</sup>. dmol<sup>-1</sup>, respectively. The CD spectrum of the tetramer or of the native



**Figure 1.** Far-ultraviolet CD spectra of the native dimeric, tetrameric, and the desensitized forms of the mung bean nucleotide pyrophosphatase. The spectra were recorded at 25°C in 1 ml of 0.1M sodium phosphate buffer, pH 7.4 containing 67.5 µg of the native enzyme, 460 µg of the tetramer or 54 µg of the desensitized enzyme. The spectrum of the desensitized enzyme plus AMP was identical to that of the desensitized enzyme alone.

dimer in the presence of AMP showed no characteristic features suggesting that AMP was either masking the spectrum or the tetramer had very low helical content (figure 1). The presence of bound *p*-hydroxymercuribenzoate in the monomeric form of the enzyme interfered with the recording of the CD spectrum (not shown in the figure). The desensitized dimer obtained by passage through Blue Sepharose showed two troughs at 210 and 222 nm with mean residue ellipticity value of  $-6,000$  deg.  $\text{cm}^2 \text{dmol}^{-1}$  at both the wavelengths (figure 1). Addition of AMP had no effect on the CD spectrum of the desensitized enzyme. The helical content of the four enzyme forms, viz., native dimer, monomer, tetramer and the desensitized forms of the mung bean nucleotide pyrophosphatase, calculated from mean residue ellipticity values at 208 and 222 nm are shown in table 1.

**Table 1.** Helical content of the native dimer, tetramer, monomer and the desensitized dimer forms of mung bean nucleotide pyrophosphatase.

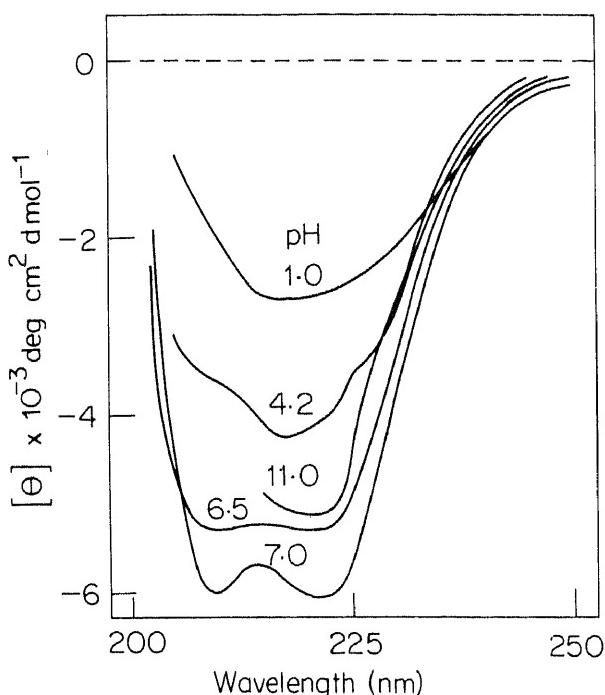
Enzyme form	Helical content (%)		
	a	b	c
Native dimer	63	63	67
Tetramer	Nil	Nil	Nil
Monomer	4	9	8
Desensitized dimer	6	12	11

a from CD data using the method of Greenfield and Fasman (1969)

b from CD data using the method of Chen and Yang (1971)

c from ORD data using the method of Chen and Yang (1971).

The ORD spectrum of native dimer (figure not given) showed a trough at 233 nm with a mean residue rotation of  $-11,000$  deg.  $\text{cm}^2 \text{dmol}^{-1}$  which decreased to  $-2,200$  deg.  $\text{cm}^2 \text{dmol}^{-1}$  on treatment of the native enzyme with  $50 \mu\text{M}$  AMP (figure 2) and to  $-3,500$  deg.  $\text{cm}^2 \text{dmol}^{-1}$  on treatment with  $0.5 \text{ mM}$  *p*-hydroxymercuribenzoate. The spectra of the tetramer and the monomer were identical with those of the native dimer in the presence of AMP or *p*-hydroxymercuribenzoate, respectively. The desensitized enzyme obtained by passage through a Blue Sepharose column (Reddy *et al.*, 1979) showed a trough at 231.5 nm and a cross-over point at 221 nm (figure 2). The mean residue rotation of the desensitized enzyme was  $-4,100$  deg.  $\text{cm}^2 \text{dmol}^{-1}$  at 231.5 nm and, unlike in the case of the native dimer, there was no change in the rotation at 231.5 nm or in the overall shape of the ORD spectrum on the addition of AMP to the desensitized enzyme. The helical contents of the various enzyme forms calculated by the method of Chen and Yang (1971) is given in table 1. It can be seen from the table that the helical content of the native dimer calculated from mean residue ellipticity values at 208 or 222 nm or from the mean residue rotation at 233 nm is in good agreement validating the equations used in these calculations. The helical content decreased markedly when the native dimer was either desensitized to AMP interactions or when AMP was bound to it.



**Figure 2.** Effect of pH on the CD spectra of the desensitized mung bean nucleotide pyrophosphatase. Enzyme concentration used was 90 µg. pH changes were effected by the addition of either dilute HCl or NaOH.

#### *Effect of pH on the conformation and the activity of the desensitized enzymes*

Figure 2 depicts the CD spectra of the desensitized enzyme as a function of pH in the range of 1-11. At pH 11.0, the CD spectrum below 215 nm could not be recorded because of the high absorbance due to the OH<sup>-</sup> ions (Visser and Blout, 1971). It can be seen that as the pH is changed from neutrality in either direction, the helical content decreases. The decrease in the acid side was more pronounced. The spectra of the desensitized enzyme at pH 4.2, 6.5, 7.0 and 11.0 returned to the spectrum at pH 7.0 suggesting that the conformational change in this pH range was reversible. However, decreasing the pH to below 4.2 caused irreversible denaturation as indicated by the observation that the original spectrum was not obtained on readjusting the pH to 7.0. The helical content at different pH values and the activity of the enzyme at that pH and after adjusting to pH 9.2, which is the optimal pH for the enzyme activity are shown in table 2. It can be seen that the recovery of activity correlates well with the recovery of the helical content.

#### *Effect of urea and sodium dodecyl sulphate on the helical content and activity of the desensitized enzyme*

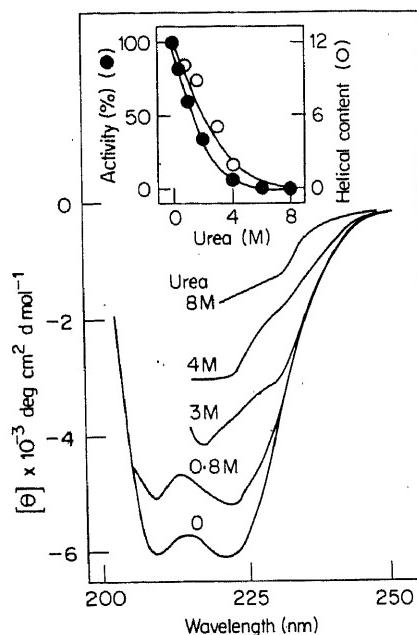
It can be seen from figure 3 that the addition of urea causes a decrease in the helical content of the desensitized enzyme. From the inset, it can be seen that the loss of helicity correlates with the loss of enzyme activity, complete loss occurring when the helical content decreases to about 2%.

**Table 2.** Effect of pH on the helical content and activity as well as stability of the desensitized enzyme.

pH	Helical content (%)	Activity	
		Before <sup>a</sup>	After adjusting to pH 9.2 <sup>b</sup>
7.5	12	27	100
6.5	9	0	90
5.6	8	0	86
4.2	6	0	80
1.0	1	0	0
9.0	12	100	100
11.0	7	0	100

<sup>a</sup> The enzyme activity was assayed at the pH indicated

<sup>b</sup> The enzyme was adjusted to pH 9.2 and assayed for activity.



**Figure 3.** Changes in the CD spectra of the desensitized nucleotide pyrophosphatase on the addition of urea. To the desensitized enzyme (55 µg) at pH 7.4, calculated amounts of urea were added to obtain 0.8, 3.0, 4.0 and 8.0 M and the spectra were recorded. The pH change on the addition of urea was marginal and a value of 7.2 was reached at 8 M urea. Volume changes consequent to the addition of urea were measured and corrected for.

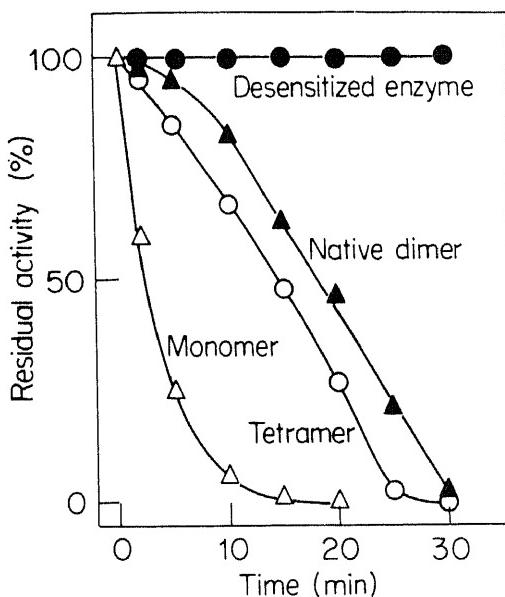
Inset represents the correlation between change in the helical content (calculated according to the method of Chen and Yang (1971) and the catalytic activity at the different concentrations of urea (0-8M). The activity in the absence of urea was normalized to 100 and the activity at the various concentrations of urea were expressed as per cent of this activity.

The effect of sodium dodecyl sulphate (0.11, 0.32 and 0.53%) on the conformation of the enzyme as monitored by the changes in the CD spectrum of the desensitized enzyme, showed a significant change in the wavelength region (205-225 nm). The absence of characteristic features in the CD spectra of the desensitized enzyme made it difficult to assign specific conformational changes, except to say that sodium dodecyl sulphate causes a change in the conformation of the protein.

#### *Stability towards denaturants*

Alterations in the structure of proteins have been monitored by comparing the profiles of the loss of catalytic activity on subjecting the enzyme to denaturation by heat and urea (Wang and Tu, 1969; Clark and Yielding, 1971; Weitzman, 1966) inactivation by EDTA (Ravindranath and Appaji Rao, 1968; Balakrishnan, *et al.*, 1974) and proteolytic digestion (Carney, *et al.*, 1978; Murakami and Murachi, 1978; Schultz and Colowick, 1969; Yamato and Murachi, 1979).

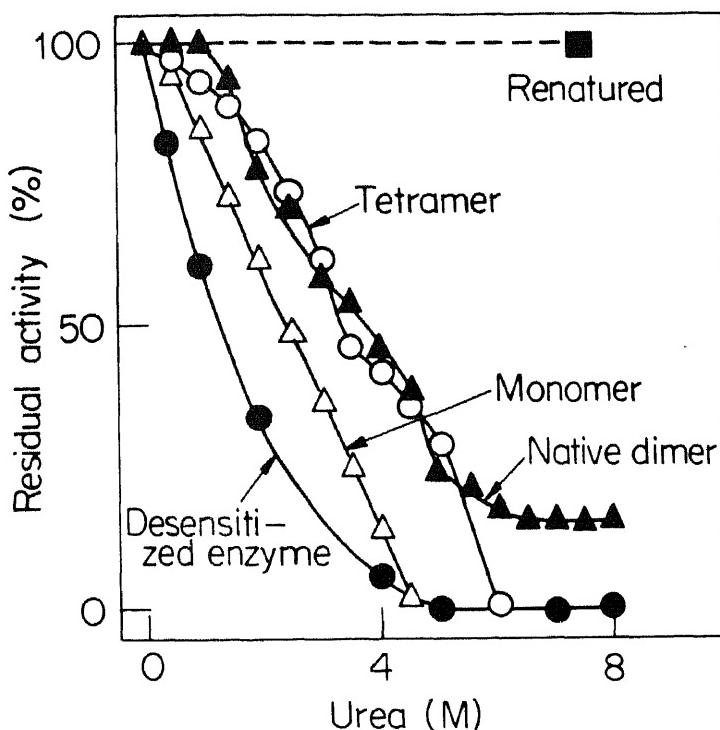
It can be seen from the figure 4 that while the Blue Sepharose-desensitized enzyme was stable at 50°C for 30 min, the native dimer, monomer and tetramer forms of the enzyme progressively lost the activity with time of preincubation at 50°C. The monomer was most susceptible to inactivation losing complete activity in 15 min., whereas 25 and 30 min were needed to completely inactivate the tetramer and the native dimer, respectively.



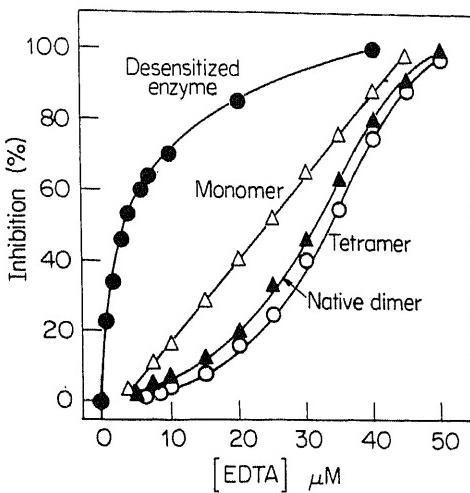
**Figure 4.** Effect of heating on the activity of the native dimer ( $\blacktriangle$ ), tetramer ( $\circ$ ), monomer ( $\Delta$ ) and the desensitized ( $\bullet$ ) forms of the mung bean nucleotide pyrophosphatase. The native dimer (12  $\mu$ g), tetramer (20  $\mu$ g), monomer (20  $\mu$ g), and the desensitized enzyme (30  $\mu$ g) were heated at 50°C. At time intervals indicated in the figure, aliquots were withdrawn and rapidly cooled to 0°C. Activity of the various forms of the enzyme kept at 0°C for 30 min and assayed at 37°C was normalized to 100 and the residual activity is expressed as per cent of this value.

The native dimeric form of the nucleotide pyrophosphatase was maximally inhibited at 6 M urea and further increase upto 8 M caused no additional inhibition (figure 5). The monomer, tetramer and the desensitized dimer were also inactivated by increasing concentrations of urea and complete loss of activity occurred at 4.5, 6 and 4 M respectively. The native dimer and the desensitized enzyme could be renatured by removal of urea whereas the monomer and the tetramer were irreversibly inactivated.

The mung bean nucleotide pyrophosphatase like several other pyrophosphatases was shown to be a zinc containing protein (Pattabhiraman *et al.*, 1964; Cabib *et al.*, 1965; Sonnino *et al.*, 1966; Corder and Lowry, 1969; Brown and Reichard, 1969; Ravindranath and Appaji Rao, 1968). The inhibition by increasing concentrations of EDTA of the activity of the native dimeric, monomeric, tetrameric and the desensitized forms of the enzyme are shown in figure 6. The desensitized enzyme was the most sensitive to EDTA inhibition as indicated by the concentration of EDTA required for 50% inhibition ( $3.5 \mu\text{M}$ ) compared to 35, 32, and  $25 \mu\text{M}$  required for the tetramer, native dimer and the monomer respectively.

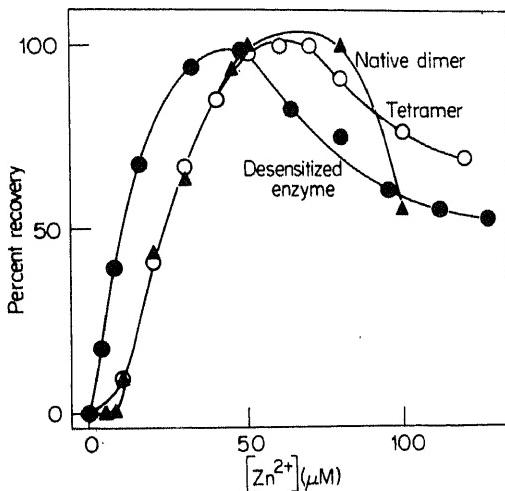


**Figure 5.** Loss of catalytic activity of native dimeric, tetrameric, monomeric and the desensitized forms of the mung bean nucleotide pyrophosphatase on treatment with urea. The four enzyme forms were assayed at different concentrations of urea (indicated in the figure) in the reaction mixture. Activity in the absence of urea was normalized to 100. The activity of the renatured enzyme obtained upon removal of urea from the native dimeric or the desensitized enzyme is shown (■) in the figure. This enzyme form obtained from the native dimer was desensitized to AMP regulation.



**Figure 6.** Effect of EDTA on the catalytic activity of the native dimer, tetramer, monomer and the desensitized forms of the mung bean nucleotide pyrophosphatase. The enzymes were assayed in reaction mixtures containing the concentrations of EDTA indicated in the figure. Activity in the absence of EDTA was normalized to 100.

The EDTA inhibition could be reversed by adding  $\text{Zn}^{2+}$ . The reactivation profiles of the native dimer, tetramer and the desensitized enzyme are shown in figure 7. However, the inhibition of the monomer could not be reversed by the addition of  $\text{Zn}^{2+}$  or any other metal ion. Very low concentrations of  $\text{Zn}^{2+}$  were sufficient to reactivate the desensitized dimer suggesting the active site was more accessible than in the case of the native dimer (table 3).



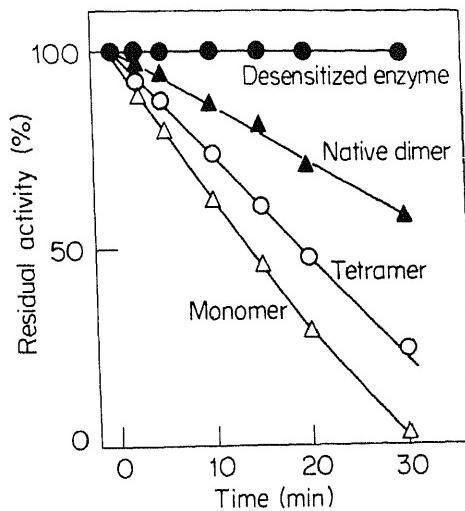
**Figure 7.** Reactivation of the apoenzyme by  $\text{Zn}^{2+}$ . The native dimeric, tetrameric and the desensitized forms (20  $\mu\text{g}$  each) of the enzyme were incubated with EDTA (1 mM) and excess EDTA removed by passage through a Sephadex G-25 column. The apoenzymes thus obtained were reactivated by assaying for activity in reaction mixtures containing concentrations of  $\text{Zn}^{2+}$  in the range of 0–150  $\mu\text{M}$ . Activity of the enzyme before the addition of EDTA was normalized to 100 and the apoenzymes had no detectable activity.

**Table 3.** Physico-chemical and kinetic properties of the native dimeric, monomeric, urea-denatured and renatured, tetrameric and the Blue Sepharose-treated forms of mung bean nucleotide pyrophosphatase

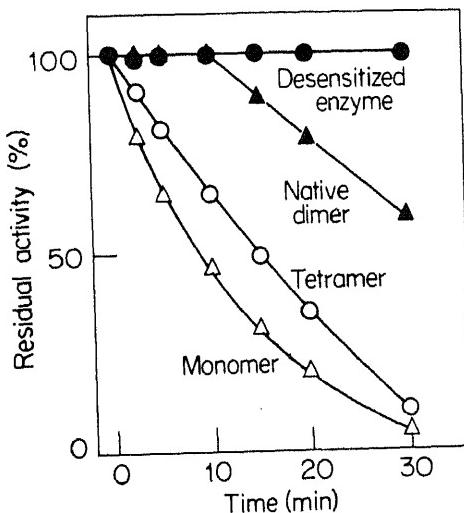
Property	Monomer	Native dimer	Urea denatured and renatured	Blue Sepharose	Tetramer
Molecular weight	35,000	65,000	65,000	65,000	130,000
Electrophoretic mobility	0.82	0.32	ND	0.63	0.79
Time course	Linear	Biphasic	Linear	Linear	Linear
pH optimum	9.4	9.2-9.4	9.2-9.4	9.3	9.4
Temperature optimum	37°C	49°C	49°C	67°C	49°C
$K_m$ for FAD (mM)	0.50	0.33	1.8	0.33	0.58
$V_{max}$ for FAD	7.0	3.3	8.0	24	2.5
AMP regulation (high affinity site)	-	+	-	-	-
AMP inhibition (low affinity site)	+	+	+	+	+
$K_i$ values for AMP (mM)	0.7	0.1 (competitive)	ND	0.1 (competitive)	0.64
$K_i$ for ADP (mM)	ND	2.2	ND	0.5-1.0	ND
$K_i$ for ATP (mM)	ND	0.26 (non-competitive)	ND	0.88 (Partial non-competitive)	ND
EDTA inhibition (conc. required for 50% inhibition) ( $\mu$ M)	25	32	ND	3.5	35
Zn <sup>2+</sup> reactivation (conc. required for 50% reactivation) ( $\mu$ M)	ND	21	ND	9	21
Urea denaturation (conc. required for 50% inhibition) (M)	2.5	3.7	ND	1.3	3.4
Ability to be reversibly denatured by urea	-	+	+	+	-
Helical content (%)	9	63	ND	12	Nil
Heat inactivation (time required for 50% inactivation at 50°C) (min)	3	18	ND	Not inactivated	14
Susceptibility to proteolysis (time required for 50% inactivation)					
(a) Trypsin (min)	10	35	ND	Not inactivated	15
(b) Chymotrypsin (min)	15	34	ND	Not inactivated	20

+ Present; - Absent; ND Not determined

It can be seen from figures 8 and 9, that the desensitized enzyme was refractory to proteolytic digestion, whereas the activities of the other forms of the enzyme were rapidly lost. The absence of the release of detectable amounts of trichloro acetic acid-soluble, ninhydrin-positive material at the end of the incubation of the



**Figure 8.** Effect of chymotrypsin on the catalytic activity of the native dimer, tetramer, monomer and the desensitized forms of the mung bean nucleotide pyrophosphatase. The native dimer (250 µg), tetramer (250 µg), monomer (250 µg), or the desensitized enzyme (100 µg) were incubated with 5 µg of chymotrypsin in Tris-HCl buffer pH 8.0 at 37°C in a total volume of 1 ml and aliquots were withdrawn at time intervals indicated and assayed for catalytic activity.



**Figure 9.** Effect of trypsin on the catalytic activity of the various forms of the enzyme. The native dimer, tetramer, monomer (250 µg each) or the desensitized enzyme (100 µg) were incubated with 5 µg of trypsin in Tris-HCl buffer, pH 8.0 at 37°C in a total volume of 1.0 ml. Aliquots portions of the reaction mixture were withdrawn at time intervals indicated in the figure and assayed for nucleotide pyrophosphatase activity.

desensitized enzyme with trypsin or chymotrypsin suggested that proteolysis was not occurring. The possibility that the desensitized enzyme was functioning as a protease inhibitor was ruled out by assaying for proteolytic activity of trypsin and chymotrypsin using casein as substrate in the presence of the desensitized enzyme. The observation that an enzyme can become protease-resistant as a consequence of a change in its structure has been documented in the case of fumarase (Yamoto and Murachi, 1979).

### Discussion

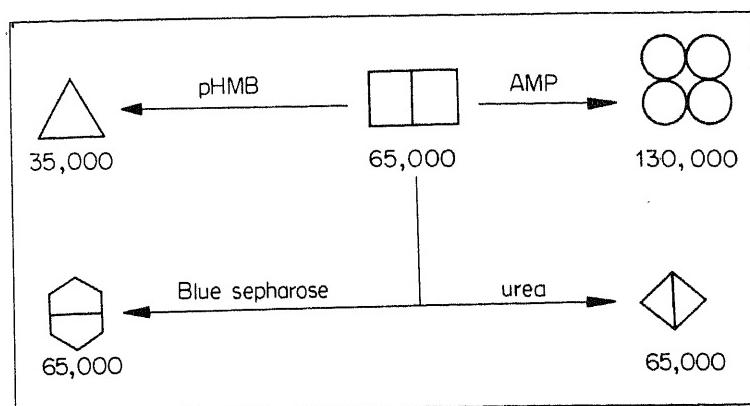
Although conformational changes in proteins on binding of ligands are well documented, comparatively less information is available on the effect of desensitization of regulatory enzymes on their conformation. The availability of native and desensitized forms of mung bean nucleotide pyrophosphatase enabled us to probe into these effects.

The addition of AMP brought a marked change in the CD spectrum of the native enzyme and also a change in its kinetic properties (decrease in  $V_{max}$  from 3.3 to 2.5, increase in  $K_m$  from 0.25 mM to 0.58 mM, loss of inhibition at low concentrations of AMP and the loss of ability to be reversibly denatured by urea). The Blue Sepharose desensitized enzyme showed difference in its kinetic properties and the CD spectrum when compared with the native enzyme ( $V_{max}$  increased from 3.3 to 24,  $K_m$  remained unaltered, temperature optimum increased from 49° to 67°C and inhibition by low concentrations of AMP was lost). The desensitization of the high-affinity binding site for AMP was confirmed by the absence of any change in the CD spectrum of the desensitized enzyme on the addition of AMP. In the case of rabbit muscle phosphorylase, small differences in the conformation were observed when phosphorylase *b* was converted to phosphorylase *a* (Graves and Wang, 1972). Changes in the catalytic activity in the absence of regulators, decrease in solubility and stability to cold denaturation were also observed (Graves and Wang, 1972). The conformational changes in the desensitized enzyme brought about by changes in the pH suggested that there was good correlation between the helical content and activity.

The stability of the desensitized enzyme compared to that of the native enzyme showed that using certain parameters, the stability was enhanced but with others it decreased. The desensitized enzyme was more stable than the native enzyme towards heat similar to the observation with citrate synthase (Weitzman, 1966), phosphorylase *b* (Wang and Tu, 1969) and glutamate dehydrogenase (Clark and Yielding, 1971). The stability to proteolytic digestion markedly changed on desensitization, reminiscent of the observation with phosphorylase *b* (Carney *et al.*, 1978).

While the sensitivity to urea denaturation was decreased in the case of citrate synthase (Weitzman, 1966) and phosphorylase *b* (Wang and Tu, 1969), the sensitivity was increased in the case of nucleotide pyrophosphatase.

Based on the results presented in this paper and from our earlier work on the enzyme (Ravindranath and Appaji Rao, 1968; 1969; Balakrishnan *et al.*, 1974, 1975, 1977; Reddy *et al.*, 1979), we postulate the following model to explain the regulation of mung bean nucleotide pyrophosphatase by AMP (figure 10).



**Figure 10.** The model describing the mechanism of the regulation of the activity of the mung bean nucleotide pyrophosphatase.

The enzyme as isolated from the germinated seedlings was a dimer,  $\square$  (Ravindranath and Appaji Rao, 1968, 1969), which became associated to form a tetramer on the addition of AMP,  $\diamond$  (Balakrishnan *et al.*, 1975) and was dissociated into a monomer by the addition of *p*-hydroxymercuribenzoate,  $\Delta$  (Balakrishnan *et al.*, 1977). Urea denaturation and renaturation by the removal of urea, resulted in the conversion of the native dimer to another dimeric form with an altered conformation,  $\lozenge$  (Balakrishnan *et al.*, 1974). The enzyme purified by using Blue Sepharose column, resulted in the isolation of yet another dimeric form,  $\heartsuit$  (Reddy *et al.*, 1979). All the enzyme forms were catalytically active but the monomer, tetramer, urea-denatured-renatured dimer and the Blue Sepharose-dimer were insensitive to the allosteric regulation by AMP, unlike the native dimer. The physicochemical and kinetic properties of these various forms of mung bean nucleotide pyrophosphatase are listed in table 3 and lends support to the model (figure 10) proposed for AMP regulation.

The native dimer catalyzed the hydrolysis of FAD with an initial fast rate followed by a second slower rate. This observation suggested that the enzyme was being converted to another stable form during the course of the reaction. Addition of AMP ( $6 \mu\text{M}$ ) before the start of the reaction abolished the initial fast rate and the reaction now followed the second slower rate (Ravindranath and Appaji Rao, 1969). Concentrations of AMP below  $1 \mu\text{M}$  were without effect and in between  $6$  and  $40 \mu\text{M}$  inhibited the enzyme activity to a constant value of 20%. Increasing the concentration of AMP above  $40 \mu\text{M}$  inhibited the enzyme activity linearly and complete inhibition was observed at about  $1 \text{ mM}$  AMP. These results suggested the possibility of two classes of sites for AMP binding on the enzyme—a high-affinity site responsible for the regulation by AMP and a low-affinity site for product inhibition. The formation of a stable enzyme form in the presence of AMP was indicated by the change observed in the electrophoretic mobility (0.33 for the native dimer and 0.79 for the tetramer). It was later shown that AMP converted the native dimer ( $M_r = 65,000$ ) into a tetramer ( $M_r = 130,000$ ) whose pH and temperature

optima were unaltered (Balakrishnan *et al.*, 1977). The  $K_m$  value of FAD was not altered but the  $V_{max}$  decreased from 3.3 to 2.5, for the native dimer and tetramer, respectively. Urea (6 M) irreversibly denatured the tetramer but could reversibly denature the native dimer. The changes in the structure were reflected by the decrease in the helical content, the time required for 50% inactivation by heat and the susceptibility to proteolytic digestion by trypsin and chymotrypsin, when the native dimer was converted to a tetramer by AMP (figures 1, 4, 8, 9 and tables 1 and 3).

Dissociation of the native dimer was brought about by treatment with *p*-hydroxymercuribenzoate and the resulting monomer had a  $M_r$  of 35,000 and functioned with the initial fast rate of FAD hydrolysis observed for the native dimer. The observation that low concentrations of AMP could neither inhibit the rate nor convert the monomer to a dimer or tetramer confirmed the hypothesis that the enzyme was desensitized to allosteric interactions of AMP on dissociation. The electrophoretic mobility of the monomer was 0.82 as compared to 0.32 for the native dimer (table 3). Although the pH optima for both the enzymes were identical, they differed in their temperature optima ( $37^\circ\text{C}$  and  $49^\circ\text{C}$ , for the monomer and native dimer, respectively), suggesting that dissociation had made the enzyme more unstable. This conclusion was reaffirmed by the observations that there was a decrease in the helical content; time required for 50% inactivation by heat at  $50^\circ\text{C}$  and susceptibility to proteolytic digestion by trypsin and chymotrypsin of the enzyme on dissociation (table 3). The  $K_m$  values for both the forms were same but they differed in their  $V_{max}$  3.3 (dimer) to 7.0 (monomer). Our attempts at interconverting the monomeric, native dimeric and tetrameric forms of the enzyme were unsuccessful.

An altered dimeric form was obtained when the native one was subjected to urea (6 M) denaturation and renaturation by the removal of urea. The altered form had  $M_r$  of 65,000, catalyzed the FAD hydrolysis with a linear time-course and low concentrations of AMP could not convert this form to a tetramer. There was a concomitant increase in the  $K_m$  and  $V_{max}$  values of the altered enzyme. Sodium dodecyl sulphate gel electrophoreses of the native dimer, tetramer, monomer and the urea-denatured-renatured form elicited a single band ( $M_r$  35,000) suggesting that all the enzyme forms hitherto mentioned were made up of identical subunits.

Yet another method of desensitizing the native dimer is by its interaction with Cibacron Blue F3GA. This interaction had no effect on the molecular weight and pH optimum, but increased the  $V_{max}$  from 3.2 to 24 and the temperature optimum  $49^\circ\text{C}$  to  $67^\circ\text{C}$ . A similar increase of  $V_{max}$  on desensitization of regulatory enzymes is well documented with other allosteric enzymes (Gerhart and Pardee, 1962; Mangiarotti and Pontremoli, 1963). The altered conformation of this desensitized dimer was indicated by its refractiveness to inactivation by heating at  $50^\circ\text{C}$  for 30 min. and proteolytic digestion by trypsin and chymotrypsin (figures 4, 8, 9 and table 3). In contrast to these observations, there was a decrease in the concentrations required for 50% inhibition by urea and EDTA (3.6 M and  $32 \mu\text{M}$  for the native dimer and  $1.3 \text{ M}$  and  $3.5 \mu\text{M}$  for the desensitized enzyme, respectively).

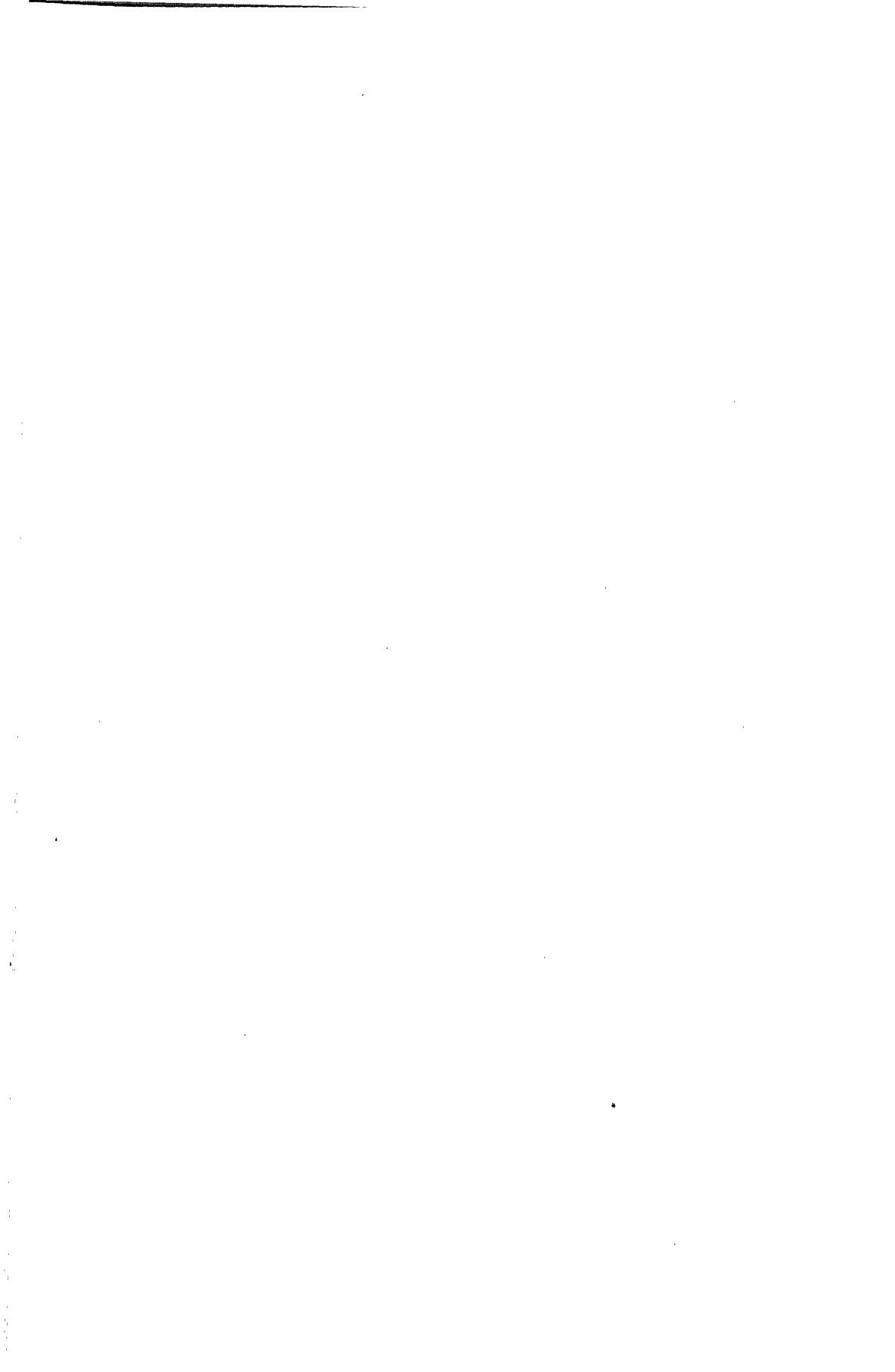
The model (figure 10) highlights the regulation of mung bean nucleotide pyrophosphatase by AMP. Although the monomer, desensitized dimer and tetramer have lost

the ability to interact with low concentrations of AMP, the site for product inhibition is still retained by all of them indicating that they function by similar catalytic mechanism.

The results emphasize that multiple mechanisms are operative in desensitization of regulatory proteins.

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## Spectroscopic studies on the denaturation of papain solubilized and Triton X-100-solubilized glucoamylase from rabbit small intestine.

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MS received 14 January 1980; revised 2 August 1980

**Abstract.** Intestinal brush border proteins consist of an enzymatically active hydrophilic moiety attached to a hydrophobic tail. Papain dissociates the hydrophilic part by cleaving off the hydrophobic tail, whereas the detergent Triton X-100 solubilizes the whole molecule. Denaturation by 8 M urea or 4 M guanidinium chloride does not alter the structure of the papain-solubilized enzyme. An appreciable alteration of the structure of detergent-solubilized enzyme was observed on denaturation. The difference spectra of Triton X-100 (1%)-solubilized enzyme and its urea denatured form shifts and intensifies, with increase in the concentration of the denaturant with an isobestic point at 252 nm. A new band at 280 nm also appears at 4 M urea concentration. Papain-solubilized glucoamylase has an  $\alpha$ -helical conformation in solution unlike the detergent-solubilized fraction. An elongated structure for the papain-solubilized enzyme is inferred from the urea denaturation studies and from molecular weight determinations.

**Keywords.** Rabbit small intestine; glucoamylase; papain denaturation; Triton X-100; difference spectra.

### Introduction

Glucoamylase (EC 3.2.1.3) is an exoenzyme that releases glucose residues directly from starch. Glucoamylase of the small intestine localized in the brush border, has been purified to varying extents from different animal species (Seetharam *et al.*, 1970, Schlegel-Haueter *et al.*, 1972; Kelly and Alpers, 1973) and to homogeneity from rabbit (Sivakami and Radhakrishnan, 1973).

In general, the disaccharidases and glucoamylase have been studied after solubilization from the brush border membrane by the proteolytic action of papain which cleaves the hydrophobic moiety from the solubilized hydrophilic moiety (Maroux and Louvard, 1976). Recently, detergents have been used for solubilizing the sucrase-isomaltase (Sigrist *et al.*, 1975) and alkaline phosphatase (Colbeau and Maroux, 1978). The detergent and papain-solubilized forms of sucrase-isomaltase are identical enzymatically but differ in their tendency to aggregate (Sigrist *et al.*, 1975). Now, it is well established that the detergent solubilizes the 'whole' enzyme by acting on the membrane-embedded hydrophobic part and this interaction appears to be dependent on the critical micellar concentration of the detergent (Helenius and Simons, 1975).

In our earlier attempts to determine the molecular weight of papain-solubilized glucoamylase from rabbit small intestine, an anomalous behaviour of the enzyme on gel filtration systems was noticed. The estimate of the molecular weight obtained

by gel filtration was at least 5 times larger than the value obtained in the analytical ultracentrifuge (Sivakami and Radhakrishnan, 1978). An explanation for this discrepancy was not then readily available.

Preliminary experiments have indicated that glucoamylase can be effectively solubilized using Triton X-100 (Nirmala Murthy, 1978). The Triton-solubilized enzyme is apparently different from the papain-solubilized enzyme in its stability to heat and denaturants like urea and guanidinium chloride (Nirmala Murthy, 1978). Spectral studies on the two forms of the enzyme were therefore initiated with a view to detect any differences in the state of aggregation and shape and to offer a possible explanation for the observed anomaly in the molecular weight of the enzyme.

### Materials and methods

All the reagents used here are of analytical grade. Urea and guanidinium chloride are of ultrapure grade obtained from Schwarz-Mann, Orangeburg, New York, USA. Papain was obtained from Sigma Chemical Company, St. Louis, Missouri, USA and Triton X-100 used was of scintillation grade from Eastman Kodak, Rochester, New York, USA. Glucoamylase was solubilized using papain or Triton X-100 and subsequently purified by affinity chromatography on Sephadex G-200 columns (Sivakami and Radhakrishnan, 1973). A 20% homogenate of the intestinal mucosal scrapings was centrifuged at 12,000 g for 30 min in a refrigerated centrifuge, model MB 20 (MB Corporation, Bombay) at 4°C. The particulate fraction was suspended in 0.01 M potassium phosphate buffer pH 7.0 to get a final protein level of about 10 mg/ml. This fraction was incubated with crystalline papain at a papain: pellet protein ratio of 1:100 for 60 min at 37°C, at the end of which it was chilled and centrifuged at 25,000 g for 3 h in a refrigerated centrifuge at 4°C. Papain was removed from the enzyme during the specific affinity procedure using Sephadex G-200 (Sivakami and Radhakrishnan, 1973). For solubilization with Triton X-100, the homogenate in the phosphate buffer was adjusted to a protein concentration of 6 mg/ml, and incubated at 4°C, for 90 min, in the presence of 1% Triton X-100 and 0.01 mM NaCl. At the end of the incubation, the mixture was centrifuged at 25,000 g for 3 h. The enzyme in the supernatant was purified by the same affinity technique under identical conditions as was used for the papain-solubilized enzyme. Throughout the purification and the subsequent analysis, 1% Triton X-100 was maintained. The Triton enzyme was apparently free of contamination as judged by polyacrylamide disc gel electrophoresis (Nirmala Murthy, 1978 and figure 1). Maltase and gluco-

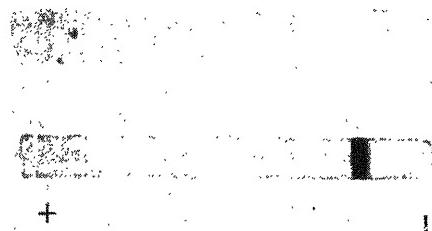


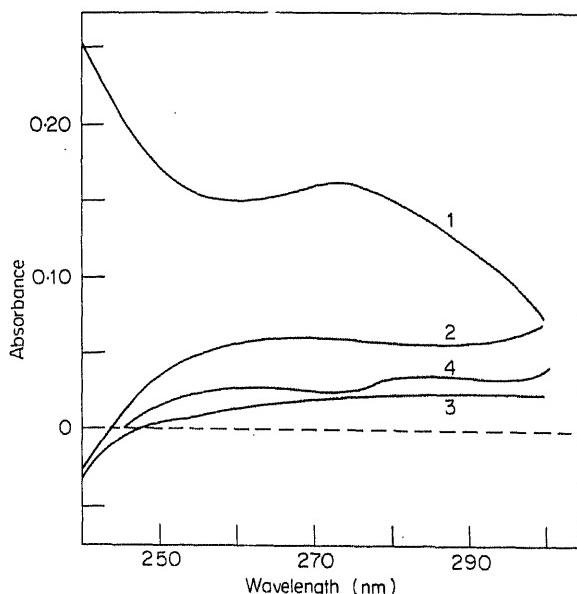
Figure 1. Polyacrylamide gel electrophoresis of Triton X-100 solubilized glucoamylase

amylase activities were determined by measuring the glucose formed by the glucose oxidase-peroxidase procedure of Dahlqvist (1964), as described earlier (Seetharam *et al.*, 1970). Protein was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Spectral measurements were carried out in 0.5 ml cuvettes using a Shimadzu UV-200S double beam spectrometer. The enzyme was allowed to equilibrate with urea and guanidinium chloride for about 1 h before the spectral measurements were made. However, the denaturation was found to be completed within 1 min after the addition of the denaturant. Difference spectra were corrected for the absorption of the urea and guanidinium chloride under similar conditions. Since Triton X-100 was present in equal amounts in both the reference and sample cuvettes, any contribution by it to the protein absorption spectra was cancelled out. By varying the concentration of both Triton-X-100 and the protein, it was also observed that the spectral properties of free Triton X-100 and protein bound Triton X-100 were the same. Circular dichroic measurements were carried out in a Jasco J-20 spectropolarimeter.

### Results and discussion

Figure 2 shows the UV absorption spectra in the range 240-300 nm of the papain-solubilized enzyme (papain-free). The enzyme was characterized by a typical broad

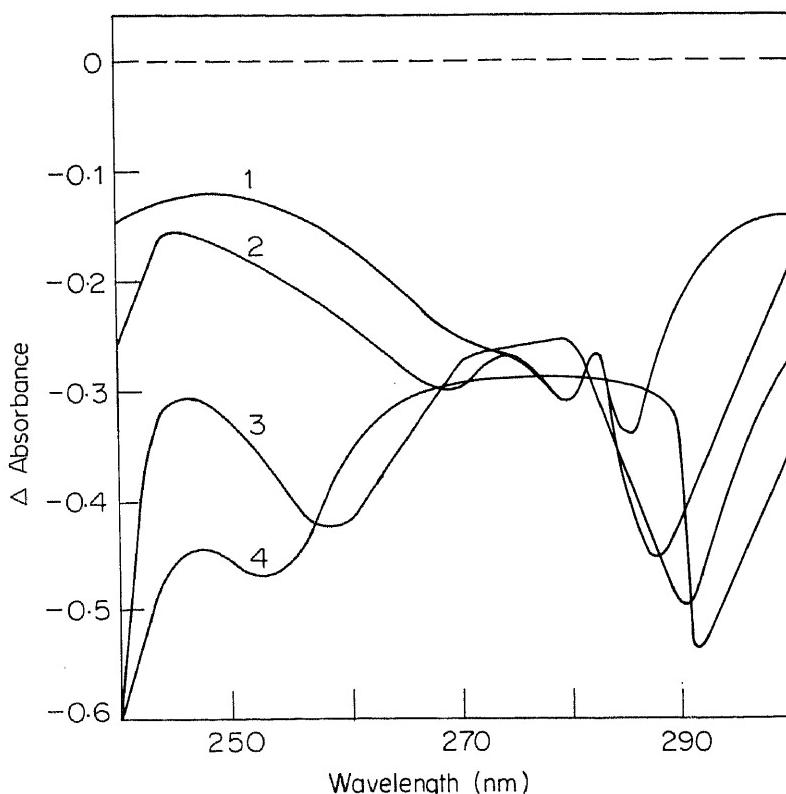


**Figure 2.** Ultraviolet spectra of papain-solubilized enzyme and the difference spectra of the enzyme in 8 M urea and 4 M guanidinium chloride. During the analysis of difference spectra, reference cuvette contained the enzyme and the sample cuvette contained enzyme and the denaturant.

1. Enzyme 40  $\mu$ g;
2. The difference spectra of the enzyme (40  $\mu$ g) + 8 M urea;
3. Enzyme 20 and 10  $\mu$ g + 8 M urea;
4. Enzyme 40  $\mu$ g + 4 M guanidinium chloride.

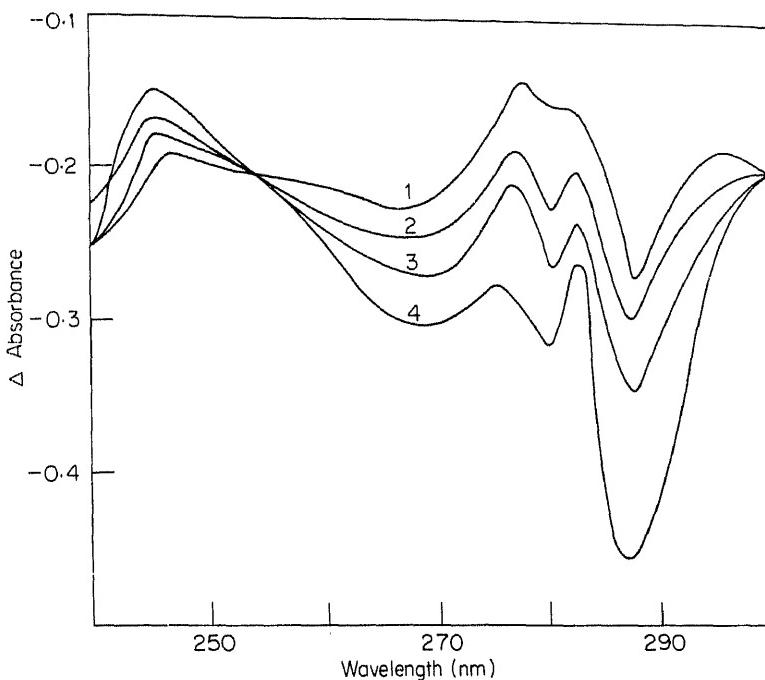
absorption peak at 272 nm contributed by the aromatic amino acid residues present in the enzyme. Phenylalanine and tyrosine were present to the extent of 69.2 mol/mol of the enzyme (Sivakami and Radhakrishnan, 1978). However, when this enzyme was subjected to denaturation with 8 M urea or 4 M guanidinium chloride, no significant change was observed as shown in the difference spectra (figure 2). Change in absorbance was considerable only when a higher concentration of enzyme was used (40  $\mu$ g). The absence of any appreciable change on denaturation suggests that the aromatic amino acid residues may not be buried in a hydrophobic region as in a globular protein.

Quite a different result was obtained when the Triton X-100 solubilized enzyme was denatured with 8 M urea (figure 3). It should be mentioned here that the concentration of Triton X-100 (1%) in aqueous medium used in these experiments was higher than its critical micellar concentration value. Therefore, the difference spectra were obtained by subtracting the contribution of the change in absorbance arising due to the disruption of the Triton micelle itself by urea (Helenius and Simons, 1975). With increase in the concentration of the protein, the large negative



**Figure 3.** Spectra of the Triton X-100 solubilized enzyme denatured with 8 M urea. Reference cuvette contained the enzyme (1, 10  $\mu$ g; 2, 20  $\mu$ g; 3, 40  $\mu$ g; 4, 80  $\mu$ g protein) and the sample cuvette contained the enzyme and 8 M urea.

band at 285 nm shifted towards a higher wavelength. Moreover, at lower concentrations of the protein (10 and 20  $\mu\text{g}$ ) a new band appeared at 280 nm. The difference spectrum was also characterized by a broad band at a lower wavelength which shifted and intensified with increase in the level of the protein. When the denaturation of the enzyme (20  $\mu\text{g}$ ) was carried out at different concentrations of urea, the band at 280 nm was found to be distinct at higher concentrations of urea (figure 4). Also,



**Figure 4.** The difference spectra of the Triton X-100 solubilized enzyme (20  $\mu\text{g}$  protein) after denaturation with different concentrations of urea (1, 2 M; 2, 4 M; 3, 6 M; and 4, 8 M urea). Reference cuvette contained enzyme and the sample cuvette contained enzyme and urea.

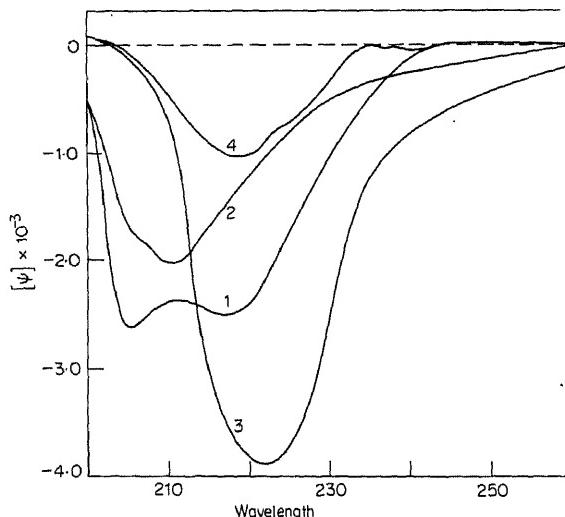
an isobestic point at 252 nm was observed indicating that one type of denatured species is present in the solution (see figure 4). Similar results were also obtained during denaturation by 4 M guanidinium chloride (not shown). All these data suggested that the enzyme in the Triton X-100 medium had a structure, the denaturation of which exposed the aromatic amino acid residues to the solvent. We would like to point out that most proteins preserve their tertiary structure in the presence of high concentration of Triton X-100 and it usually does not appear to induce conformational change in proteins leading to loss of their biological properties (Helenius and Simons, 1975). It is noteworthy that most of the proteins give a large negative band in the difference spectra upon denaturation with 8 M urea (Herskovits, 1967). However, any change in the spectra arising due to the change in concentration of the enzyme may be attributed to the aggregation of the molecule which is possible in the presence of non-ionic detergents like Triton X-100 (Tanford *et al.*, 1974; Simons *et al.*, 1973).

Hence, a Triton-free form of the enzyme was purified by washing the column thoroughly with Triton-free buffer before elution of the enzyme. The Triton free-enzyme was expected to form an aggregate.

However, the addition of 8 M urea fails to bring about any appreciable change in the organization of the enzyme aggregate, as noticed by difference spectrophotometry. Only a broad negative band of low intensity around 290 nm was observed and did not change with the concentration of the enzyme aggregate. To verify whether the spectra of the Triton-solubilized enzyme were due to the conformational effects of Triton X-100 on an enzyme form similar to that solubilized by papain, we carried out the urea denaturation study of the papain-solubilized enzyme in the presence of 1% Triton. The difference spectra did not show any band in the region of 250 to 290 nm, although there was a small negative band around 300 nm. The 8 M urea induced denaturation did not cause any significant structural alteration of the papain-solubilized enzyme in the presence of 1% Triton X-100.

All these results indicated that there was a gross structural difference of the enzyme when solubilized by using either papain or Triton. It is interesting to note that a small hydrophobic tail of the enzyme might contribute significantly to the overall conformation of the enzyme. When the difference spectra of the papain-solubilized enzyme at pH 12 was compared with that at neutral pH, the nature of the curve was found to be similar to that obtained with free tyrosine. However, no significant change was observed in alkali-induced difference spectra of Triton-solubilized enzyme (not shown).

To confirm the above pattern of results we measured the circular dichroic (CD) spectra of the urea denatured enzyme solubilized by Triton X-100 and papain respectively. The papain-solubilized enzyme had two negative CD bands, one around 218 nm and the other at 205 nm typical for  $\alpha$ -helical conformation (figure 5)



**Figure 5.** Circular dichroism spectra of papain-solubilized enzyme, (1); 8 M urea denatured papain-solubilized enzyme (2); Triton X-100 solubilized enzyme (3); 8 M urea denatured Triton X-100 solubilized enzyme (4).

(Greenfield and Fasman, 1969), whereas the Triton-solubilized fraction was characterized by one negative CD band at 222 nm. Urea denaturation caused a greater disruption of the structure of detergent-solubilized fraction than that of the papain solubilized enzyme as expected.

It was mentioned earlier that the papain-solubilized glucoamylase had an estimated molecular weight five times more by gel filtration than that obtained through ultracentrifugation. This discrepancy might arise due to the aggregation of the molecule or if the enzyme has an elongated structure. Urea denaturation data suggested that the molecule might be elongated without much folding, resulting in the enzyme eluting on gel filtration system earlier than it should (Sivakami and Radhakrishnan, 1978). On the other hand, the detergent-solubilized glucoamylase appeared to have a typical three-dimensional folding with buried aromatic amino acid residues which are vulnerable to urea denaturation. Further studies on the structure of the enzyme above and below the critical micellar concentration of the detergent and the nature of interaction of the detergent micelle with the enzyme are in progress.

### Acknowledgements

The authors wish to thank Prof. A. N. Radhakrishnan for his kind co-operation and helpful suggestions during the course of this investigation.

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## Lipid requirements for axenic cultivation of *Entamoeba histolytica*

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MS received 11 March 1980

**Abstract.** Fatty acids, cholesterol and glucose present in axenic medium are utilized by growing *Entamoeba histolytica* but the amoeba is unable to synthesize cholesterol from [ $^14\text{C}$ ] glucose although the label is incorporated into the fatty acids and non-saponifiable fractions of the organism. Exogenously-added sonicated dispersions of cholesterol,  $\beta$ -sitosterol, lanosterol, lecithin and lauric, palmitic, linoleic and stearic acids are ingested by the amoebae with subsequent loss in amoeboid movement. After a few hours the movement is regained. Cholesterol, lecithin and the fatty acids stimulate amoebic multiplication but are unable to replace serum in the medium either singly or in combination.

**Keywords.** *Entamoeba histolytica*; axenic; cholesterol; lecithin; fatty acids; trophozoite-multiplication.

### Introduction

Cholesterol has been found to convert attenuated and non-invasive strains of *Entamoeba histolytica* into invasive forms (Biagi *et al.*, 1962; Bos and Van Griend, 1977; Das and Ghoshal, 1975; Das and Singh, 1965; Lwoff, 1951; Sharma, 1959; Singh, 1959; Singh *et al.*, 1971; Meerovitch and Ghadirian, 1978). This observation has necessitated a detailed and systematic study of the lipid requirements of the organism.

Several workers observed the growth-promoting effect of cholesterol and dihydro-cholesterol on *E. histolytica* growing in the presence of bacteria. Neutral lipid and free cholesterol fractions of horse serum (one of the constituents of the Shaffer-Frye medium) when coated on the surfaces of culture tubes, stimulated multiplication of *E. histolytica* (NRS strain) growing in the presence of *Fusobacterium symbiosus*, while the phospholipid fraction or cholesterol esters did not promote growth (Cedillos *et al.*, 1961; Lwoff, 1951; Rees *et al.*, 1944). Although serum, a constituent of the medium used for axenic cultivation of *E. histolytica*, is a source of lecithin, cholesterol and fatty acids, the effect of these lipids on trophozoite-multiplication under axenic conditions has not been studied. In earlier communications from our laboratory it was reported that a sonicated dispersion of the lipids of several human intestinal bacteria stimulated trophozoite-multiplication of *E. histolytica* 200:NIH under axenic

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CDRI Communication No. 2516.

Abbreviations used: TLC, thin layer chromatography; GLC, gas liquid chromatography.

conditions (Rai *et al.*, 1978a) and free fatty acid, non-saponifiable and neutral lipid fractions of *Streptococcus faecalis* stimulated growth but the phospholipid fraction of the bacteria, which was mainly composed of phosphatidyl ethanolamine, phosphatidic acid and diphosphatidyl glycerol, and cephalin from egg (phosphatidyl ethanolamine + phosphatidyl serine) inhibited amoebic multiplication (Rai *et al.*, 1978b). These workers also found that  $^{14}\text{C}$ -labelled non-saponifiable fractions of lipids of the bacteria (obtained by culturing the bacteria in the presence of [ $\text{U-}^{14}\text{C}$ ]-glucose) were incorporated into the amoebic cells and converted into cholesterol.

Therefore, with a view to evaluate the lipid requirements of *E. histolytica* 200:NIH, we have studied utilization by the amoeba of glucose, cholesterol and fatty acids, present in axenic medium (Singh *et al.*, 1973) and the effect of these lipids, when added to the medium as sonicated dispersions, on trophozoite-multiplication.

### Materials and Methods

#### Materials

Cholesterol was purchased from Centron Laboratories, Bombay; egg lecithin was from Biochemicals Unit, V.P. Chest Institute, New Delhi; ergosterol, lauric, palmitic, linoleic and stearic acids were from Nutritional Biochemicals Corporation, Cleveland, USA. Lanosterol was isolated from wool grease according to Downing *et al.* (1960).  $\beta$ -Sitosterol was a kind gift from Dr S. K. Nigam, National Botanical Research Institute, Lucknow.

#### Parasite

200:NIH strain of *E. histolytica*, received from Dr. L. S. Diamond, National Institute of Health, Bethesda, MD, USA was maintained regularly in TPS-2 medium (Singh *et al.*, 1973). This is a modified TPS-1 medium (Diamond, 1968) containing cysteine hydrochloride but without ascorbic acid. Axenic cultivation of the amoeba was carried out in sterile screw-capped tubes (16×125 mm), 1 ml of the medium containing 10,000 trophozoites was inoculated into each tube containing 9 ml of fresh medium and the tubes were incubated at 37°C in upright position. Amoebic growth was followed by counting the number of trophozoites in a haemo-cytometer under an inverted microscope.

#### Dispersion of lipids

The lipids were dissolved in a minimum amount of alcohol, injected into deionized water under constant bubbling of  $\text{N}_2$  and the suspensions were sonicated at 20 Kc/sec, energy output 1.5 amp for 20 min at 20°C in a Mullard Ultrasonic Generator. The sonicated dispersions were filtered through Millipore membrane filters ( $0.22\ \mu$ ). Lecithin, cholesterol and fatty acids were assayed in the filtrates as described under analytical procedures and 0.1-1.0 ml aliquots were aseptically added to culture tubes before inoculation to give the concentrations specified for each experiment.

#### Harvesting of trophozoites

After incubation, the culture tubes were centrifuged at 900 g for 20 min at 0°C, the pellets of amoebae were combined and washed three times with normal saline.

Glucose was estimated in fresh media and culture supernatants obtained after harvesting the cells according to Nelson (1944).

#### *Incorporation of [U-<sup>14</sup>C]-glucose*

Appropriate amounts of [ $U-^{14}C$ ]-glucose (specific activity 2.5 mCi/mmol, Isotope Division, Bhabha Atomic Research Centre, Bombay) were added to the medium before sterilization, so that the radioactivity in each tube (10 ml) was 2.5  $\mu$ Ci. After inoculation and incubation, the trophozoites were harvested, repeatedly washed with fresh medium (non-radioactive) till no radioactivity was detected in the washings. Aliquots of fresh media or combined culture-supernatants and washings were spotted on strips of Whatman No. 1 filter paper. Pellets of trophozoites were digested with 0.5 ml hyamine hydroxide (Packard Instruments Company, Zurich, Switzerland) at 60°C overnight. For the detection of radioactivity in the liberated CO<sub>2</sub>, the amoebae were grown in screw-capped tubes provided with a U shaped side arm (2-3 mm diameter) just above the level of the medium in the tube and the side arm was filled with 0.5 ml hyamine hydroxide to trap the CO<sub>2</sub>. After inoculation and incubation, the hyamine hydroxide was withdrawn with a microsyringe.

#### **Analytical procedures**

Fresh media or culture supernatants obtained after harvesting the trophozoites were first lyophilized, and then extracted with chloroform:methanol (2:1 v/v) while the pellets of trophozoites were directly extracted according to Folch *et al.* (1951). Cholesterol and phosphorus in the lipids were estimated according to the method of Zlatkis *et al.* (1953) and Wagner *et al.* (1962) respectively.

The lipid samples were saponified with alcoholic sodium hydroxide (10% w/v) and the liberated fatty acids were esterified with diazomethane, according to James (1960). Gas liquid chromatography (GLC) of the methyl esters was carried out in 15% DEGS column in a Perkin-Elmer instrument through the kind courtesy of Professor S. M. Osman, Chemistry Department, Aligarh Muslim University, Aligarh, and Dr K. K. G. Menon, Hindustan Lever Research Centre, Bombay. Fatty acid composition was determined on the basis of area of individual peaks which were identified by comparison of retention time with authentic methyl esters.

Radioactive samples were taken in 15 ml of scintillation fluid (0.4% PPO, 0.1% POPOP in freshly distilled toluene diluted with equal volume of ethoxyethanol) and the radioactivity was assayed in a liquid scintillation Spectrometer (Packard Model 300, 90% counting efficiency for  $^{14}$ C).

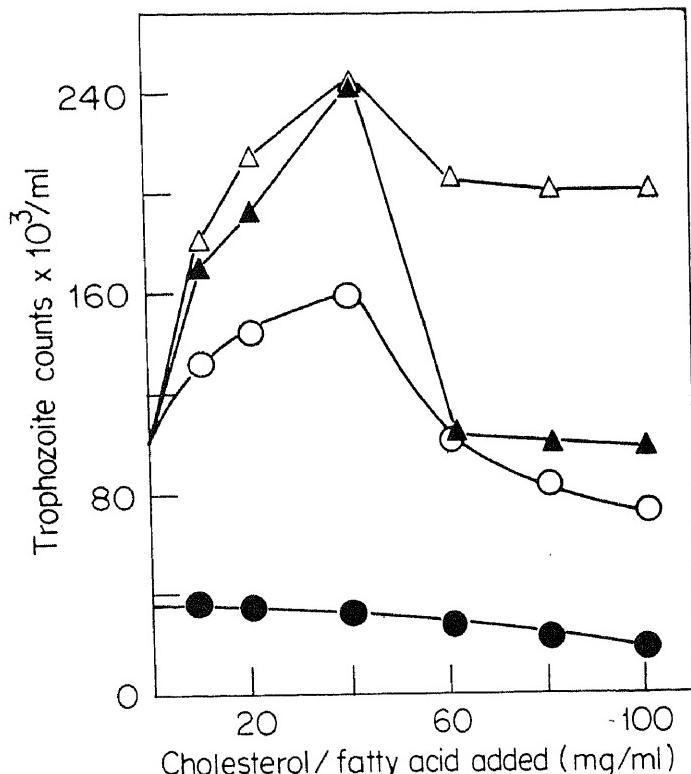
#### **Results and discussion**

Axenized *E. histolytica* 200:NIH contained about 24 mg lipid/100 million trophozoites, 30% of the lipid was phospholipid and 10% was sterol. Cholesterol was the major sterol but traces of another spot giving blue colour with orthophosphoric spray and of lower mobility than cholesterol on TLC was also present. Fatty acid composition (%) of the lipid was: lauric, 9.5; myristic, 13.9; palmitic, 11.8; stearic, 10.5; oleic, 5.0; linoleic 1.7 and unidentified fatty acids equivalent to chain length C20:4, 14.8 and C27:5, 39.6. The axenic culture medium used in this study contains 5.6 mg cholesterol/100 ml and the % composition of constituent fatty acids of the medium were:

palmitic, 30; oleic, 20 and linoleic, 50. The culture supernatant obtained after 48 h of amoebic growth contained 5.5 mg cholesterol per 100 ml and gave the following % fatty acid composition: palmitic, 20; oleic, 5 and linoleic, 75. Thus 100  $\mu\text{g}$  cholesterol/100 ml medium was utilized during amoebic growth and palmitic and oleic acids were preferentially utilized.

During growth, the amoeba consumed about 300 mg glucose per 100 ml medium. Fifty per cent of the radioactivity added as [ $\text{U}-^{14}\text{C}$ ]-glucose to the medium was recovered in the culture supernatant obtained after harvesting the trophozoites, only 0.1% of the radioactivity added to the culture was incorporated into the trophozoites and appreciable amount was detected in  $\text{CO}_2$  liberated during amoebic growth. Only 12% of the total radioactivity incorporated in the amoebae was in the lipids, but there was no incorporation in the cholesterol fraction.

TPS-2 medium supplemented with lecithin and cholesterol separately upto 40  $\mu\text{g}/\text{ml}$  stimulated amoebic multiplication to 2 and 2.45 times that of control respectively, while the addition of both lecithin and cholesterol at this concentration stimulated the amoebic multiplication to 2.5 times that of the control (figure 1).



**Figure 1.** Effect of exogenous cholesterol and/or lecithin on multiplication (96 h) *E. histolytica*.  
 Serum omitted + cholesterol and/or lecithin, ●;  
 Serum + cholesterol, ○;  
 Serum + lecithin, ▲;  
 Serum + lecithin + cholesterol, △  
 (10,000 trophozoites inoculated, 96 h incubation).

However, when lecithin or cholesterol or both were added to the medium which did not contain serum, no multiplication of trophozoites was observed. Addition of lauric, palmitic, linoleic and stearic acids to the medium stimulated amoebic growth by 4.7, 7.0, 5.0, 4.6 fold, respectively while the corresponding fold multiplication in control sets where no fatty acid was added was 3.4. With the exception of stearic acid, amoebae grown in the presence of other fatty acids were more active. The addition of cholesterol in conjunction with palmitic or linoleic or stearic acids markedly stimulated amoebic multiplication by 22, 18 and 14 fold, respectively. The amoebae were more active, bigger in size and ingested lipid micelles were seen (table 1). However, addition of lauric acid and cholesterol to the axenic medium inhibited multiplication and caused reduction in the size and activity of trophozoites.

Table 1. Effect of exogenous fatty acids and cholesterol

Fatty acid	Cholesterol	× Fold multiplication	Morphology/motility
Lauric	—	4.7	Active movement
	+	2.0	Dead amoebae; few are round; smaller size
Palmitic	—	7.0	Active movement
	+	20.0	Very active movement; bigger size; lipid micelles ingested.
Linoleic	—	5.0	Active movement
	+	18.0	Very active movement; medium size; lipid micelles ingested
Stearic	—	4.6	Sluggish
	+	14.0	Active movement
None	—	3.4	Sluggish movement
	+	11.0	Active movement

— Cholesterol not added; + 100 µg cholesterol/ml medium; 100 µg. fatty acid/ml medium 72 h incubation.

When the sonicated dispersion of cholesterol or  $\beta$ -sitosterol or lecithin was added aseptically to the trophozoite suspension the amoeboid movement increased in the first 1-3 h after which the amoebae became rounded. Active movement of trophozoites was restored after 18 h. Lanosterol did not exhibit this phenomenon while ergosterol caused lysis of the amoebae.

Utilization of glucose in the medium by *E. histolytica* (strains DKB and K9) and the incorporation of the carbon fragments of the sugar into fatty acids of the amoeba has been demonstrated by Sawyer *et al.* (1967), but this work was carried out in the presence of bacterial cells and antibiotics.

On the other hand Van Vliet *et al.* (1975) have shown that under axenic conditions *E. invadens* exhibits absolute auxotrophy for cholesterol and fatty acids. Our results show that *E. histolytica* NIH:200 utilizes [ $^3$ H]-glucose present in the axenic medium;

carbon fragments of the sugar are incorporated into fatty acids of the amoeba but there is no incorporation into cholesterol. Palmitic and oleic acids of the medium are utilized by the amoeba and converted into its constituent fatty acids. Exogenous fatty acids, when added to the medium, are ingested by the amoeba and stimulate multiplication of the trophozoites. Similarly, cholesterol of the medium is utilized by the amoeba during growth and micelles of cholesterol added to the medium are ingested by the amoeba and stimulate growth and multiplication. Thus it is evident that *E. histolytica* 200:NIH exhibits, during axenic cultivation, absolute auxotrophy for cholesterol and partial auxotrophy for fatty acids.

Latour and co-workers (1965) have shown that the phospholipid fraction of horse serum is unable to stimulate growth in *E. histolytica* (NRS strain) in the presence of *Bacteroides symbiosus*. Phospholipases are known to be present in bacteria (Van Deenen and De Hass, 1966). It is quite likely that the phospholipids added by these workers to amoeba-bacteria cultures were hydrolyzed during growth. Our results show that a sonicated dispersion of lecithin, when added to the medium, stimulated trophozoite-multiplication under axenic cultivation. Since lecithin cannot be removed from the medium without extracting other lipids and denaturing proteins and lipoproteins, the absolute requirement of lecithin in axenic cultivation of the amoeba cannot be demonstrated by reconstitution. This phospholipid is present in *E. histolytica* (Sawyer *et al.*, 1967) but it is not known if the amoeba can synthesize it from precursor molecules such as the fatty acids, glycerol, phosphate and choline.

Although, the present study has demonstrated the requirement of cholesterol, fatty acids and lecithin in axenic cultivation of *E. histolytica* 200:NIH, these lipids, either singly or in combination, cannot replace serum, which is an essential component of the axenic medium (Diamond, 1968) and is a source of these lipids. It is likely, that in addition to cholesterol, fatty acids and lecithin, carrier-proteins or lipoproteins of serum are essential for transport of lipids to amoeba and for its growth and multiplication.

### Acknowledgement

We are grateful to Mr. N. K. Verma for providing excellent technical assistance.

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## **Age-related qualitative and quantitative changes in tRNA population of rat skeletal muscle**

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MS received 21 March 1980; revised 12 May 1980

**Abstract.** Total tRNA was purified from skeletal muscle of young, adult and old female albino rats. Age-dependent variation of total tRNA was the same with respect to tRNA content and biological activity as measured by amino acid acceptor capacity. The tRNA content was more in young rats and showed a gradual decrease in the adult and old rats. The relative abundance of eleven aminoacyl-tRNAs were checked at each age and during aging. Arginyl, glutamyl and tyrosyl-tRNAs do not show any quantitative or qualitative change with age.

**Keywords.** Rat; skeletal muscle; tRNA; aging; aminoacylation; aminoacyl-tRNA fractionation.

### **Introduction**

Age-related variation in tRNA population has already been reported in lower organisms (Reitz and Sanadi, 1972; Hoffman, 1972; Hoffman and McCoy, 1974), plants (Shugart, 1972; Bick *et al.*, 1970; Mettler and Romani, 1976; Palatnik and Katz, 1977) and animals (Portugal, 1972 a, b; Dakee, 1976; Mays *et al.*, 1978). Similar investigations made by other workers revealed no change in isoacceptor species of tRNA with age (Frazer and Yang, 1972; Klee *et al.*, 1978). In general, changes are observed only in a few specific tRNAs in any system. A regulatory mechanism, was proposed for these changes in the quantity and structure of specific tRNAs in the translation process, which in turn controls cellular differentiation, development and aging.

In the present work, a study of the age-related changes in tRNA population in skeletal muscle of female albino rats was undertaken. Female rats of three age groups representing pre-reproductive (6-8 weeks), reproductive (38-40 weeks) and post-reproductive (78-80 weeks) phases of the life span were used. These groups are referred to as young, adult and old respectively. Transfer RNA was isolated from skeletal muscle to compare the total tRNA population at all the three age-groups. Following the isolation of tRNAs and aminoacyl-tRNA synthetases, transfer RNAs

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Abbreviations used: EGTA: ethyleneglycoltetraacetic acid.

were aminoacylated with 11 amino acids to assay for quantitative changes in the acceptor activity for each of these amino acids during growth and aging. Finally qualitative changes in the isoacceptor species of arginyl, glutamyl and tyrosyl-tRNAs were analyzed using DEAE-sephadex column chromatography.

### Materials and methods

Female albino rats of Wistar strain were used for the studies. Skeletal muscle from legs and arms from 8-10 rats of each age group were pooled and used as starting material to isolate total tRNAs.

#### *Isolation of total tRNA*

Transfer RNA was extracted in identical fashion from skeletal muscle of young, adult and old rats according to the method of Dure (1973) with some modification. All the steps were carried out at 4°C unless otherwise specified. The tissue was treated twice with 0.005 M phosphate buffer, pH 6.8, 0.1M KCl, 0.005M MgCl<sub>2</sub>, 0.005M EGTA before homogenization according to the method of Sherton and Wool (1974). Calcium ions are removed from the muscle by the chelating agent EGTA causing its relaxation and thus easier and efficient homogenization was achieved. Tissue was homogenized in 0.01 M Tris-HCl buffer, pH 7.5, 0.01M KCl, 0.01M MgCl<sub>2</sub>, 0.001M EDTA, 1% sodium deoxycholate and 0.001M 2-mercaptoethanol. Homogenate was centrifuged at 14,000 g for 15 min and the pellet was re-extracted. The supernatant was treated thrice with equal volume of phenol for 30 min. The aqueous layer was made 3% with potassium acetate, pH 5.0 and precipitated with 2.5 volume of ice-cold ethanol. The precipitate was recovered by centrifugation at 14,000 g for 15 min and dissolved in 0.01M Tris-HCl buffer, pH 7.5, 0.01M MgCl<sub>2</sub>, 0.25M NaCl. Sample was loaded on to DEAE-cellulose column (15 cm×1.2 cm) pre-equilibrated with the same buffer. Transfer RNA was eluted from the column using 1.0M NaCl in the same buffer. Fractions containing tRNA were pooled and tRNA was precipitated with ice-cold ethanol. Total tRNA was loaded on to a Sephadex G 100 column (75 cm×1 cm) previously equilibrated with 0.01M Tris-HCl buffer, pH 7.5, 0.4M NaCl, 0.01M MgCl<sub>2</sub> and eluted with the same buffer. This step was carried out at 25°C. The purified tRNA after gel filtration eluted in the range of standard *Escherichia coli* tRNA. The fractions were pooled and precipitated with ice cold ethanol to recover tRNA. Transfer RNA was always deacylated before aminoacylation reaction.

DNA and RNA concentration of the homogenate were estimated according to the method of Schneider (1957).

#### *Preparation of aminoacyl-tRNA synthetases*

Aminoacyl-tRNA synthetases from young, adult and old rat tissue were purified according to the method of Yang and Noveli (1971), with some modifications. Fresh tissue was homogenized in 0.01M Tris-HCl buffer, pH 7.6, 0.005M magnesium acetate, 0.01M KCl, 0.005M EDTA, 0.01M 2-mercaptoethanol and centrifuged at 14,000 g for 15 min at 4°C. The clear supernatant was centrifuged at 150,000 g for 1 h in an ultracentrifuge (Vac 601). The supernatant was applied onto a DEAE cellulose column (15 cm×1 cm) which had been previously equilibrated at 4°C with

phosphate buffer, pH 7.6, 0.001M MgCl<sub>2</sub>, 0.002M dithiotreitol and 0.005M KCl. The aminoacyl-tRNA synthetases were eluted with the same buffer containing 0.3M KCl. Fractions having synthetase activity were dialyzed against 0.05M Tris-HCl buffer, pH 7.5, 0.005M EDTA and 0.002M dithiotreitol. Aliquots (0.5 ml) were stored frozen at -20°C until use. The column step was necessary to remove endogenous tRNAs and free amino acids from the enzyme preparation. The enzyme protein was estimated according to the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

### *Aminoacetylation*

Amino acid acceptor activity of tRNA was determined by measuring the incorporation of radioactive amino acids into trichloroacetic acid insoluble material using the paper disc assay method of Mans and Novelli (1961). The reaction mixture contained 100 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 10 mM KCl, 10 mM NH<sub>4</sub>Cl, 4 mM 2-mercaptoethanol, 2 mM ATP, 1 mM CTP, 2.0 A<sub>260</sub> units of tRNA and 0.3 mg of enzyme protein (from homologous source) in a total volume of 0.3 ml [<sup>14</sup>C]-algal protein hydrolysate (0.17  $\mu$ Ci) from Bhabha Atomic Research Centre, Trombay, Bombay was used as a source of total amino acids to assay total amino acid acceptor capacity of tRNAs. The reaction was carried out for 25 min at 30°C and then terminated by trichloroacetic acid addition. The precipitate was collected and counted in a toluene-based scintillation fluid containing 4 g of 2,5-diphenyl-oxazole (PPO) and 0.1 g of 1,4-bis [2-(5-phenyloxazoyl)] benzene (POPOP) per litre of toluene.

### *Preparation of aminoacyl-tRNA*

For chromatographic studies, the total volume of the reaction mixture was scaled up. Following aminoacetylation of tRNAs for 25 min, the reaction mixture was diluted with 2.6 volumes of 0.005 M sodium acetate buffer, pH 5.0, 0.1 M NaCl and cooled in an ice-bath. The sample was adsorbed onto a small DEAE-cellulose column (1 ml bed volume) pre-equilibrated with the same buffer. Aminoacyl-tRNA was eluted with 2.0M NaCl in the same buffer and recovered by precipitation with ice-cold ethanol. The precipitate was dissolved in 0.02M sodium acetate buffer, pH 4.5, 0.50 M NaCl and 7 M urea and stored at -20°C until use. Using this procedure, [<sup>14</sup>C]-aminoacyl-tRNAs were prepared from muscle of adult and old rats and [<sup>3</sup>H] aminoacyl-tRNAs from that of young rats. Aminoacyl-tRNAs from young and adult or from young and old rats were mixed and co-chromatographed on a DEAE Sephadex column.

### *Cochromatography on DEAE-Sephadex column*

The mixed tRNA sample was applied onto a DEAE-Sephadex column (60 cm × 1 cm) and the column was developed at room temperature using a linear 140 ml sodium chloride gradient ranging from 0.50 M to 0.66 M in 0.02 M acetate buffer, pH 4.5, 7 M urea. Fractions of 0.8 ml were collected at a flow rate of 4 ml/h. Aliquot of the fractions (0.5 ml) were applied on Whatman number 1 filter paper strips (2.5 cm × 5 cm). The strips were dried under infrared lamp and washed twice with 75% ethanol containing 0.1 M NaCl to remove urea, dried and counted in a toluene based scintillation fluid as mentioned earlier.

## Results

Table 1 shows age-related variation in tRNA content (based on their absorbancy at 260 nm) of skeletal muscle. When expressed on a weight basis, 20 absorbance units at 260 nm were assumed to be equivalent to one mg of tRNA. Transfer RNA content was maximum in young tissue. It showed a gradual decrease with age, the lowest content being in old age. This decrease from the young to adult and from adult to old is quite significant when expressed as  $\mu\text{g}$  tRNA per mg of DNA which is the best parameter to express nucleic acid content of any tissue. The same pattern of variation was observed when the data was expressed per g weight of tissue, per mg RNA or per g protein.

**Table 1.** Age related variation in tRNA content of female rat muscle.

Unit of measurement	Age of rats		
	7 weeks	40 weeks	80 weeks
$\mu\text{g}$ tRNA/g wet weight	44.60 $\pm$ 0.60 <sup>a</sup>	40.00 $\pm$ 1.00 <sup>d</sup>	39.60 $\pm$ 0.50
$\mu\text{g}$ tRNA/mg DNA	67.40 $\pm$ 0.20 <sup>b</sup>	62.40 $\pm$ 0.60 <sup>c</sup>	58.80 $\pm$ 1.70
$\mu\text{g}$ tRNA/g protein	1246.00 $\pm$ 15.00 <sup>b</sup>	990.00 $\pm$ 9.80 <sup>d</sup>	970.00 $\pm$ 10.00
$\mu\text{g}$ tRNA/mg RNA	61.10 $\pm$ 1.40 <sup>d</sup>	58.80 $\pm$ 1.50 <sup>b</sup>	45.80 $\pm$ 0.30

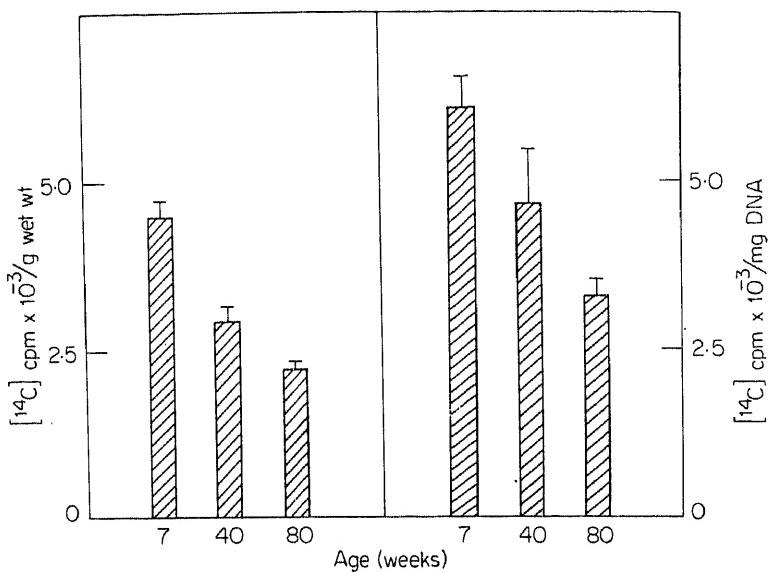
'p' value is calculated between young and adult and adult and old.

<sup>a</sup>,  $p < 0.01$ ; <sup>b</sup>,  $p < 0.001$ ; <sup>c</sup>,  $p < 0.05$ ; <sup>d</sup>, not significant.

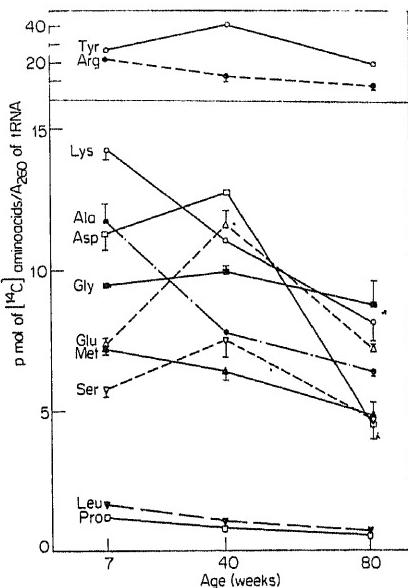
The variation in the total amino acid acceptor capacity of tRNAs from young, adult and old tissues was measured using [ $^{14}\text{C}$ ]-algal protein hydrolysate as the source of labelled amino acids and aminoacyl-tRNA synthetases from homologous sources. Total amino acid acceptor activity of tRNAs showed a similar pattern of variation with age as found in the case of total tRNA content (figure 1). This showed that total tRNA isolated from each tissue retained biological activity throughout the experimentation. The acceptor activity of tRNA was highest in the young and it decreased with age reaching the lowest levels in the old. When expressed per mg DNA, a similar variation pattern was observed.

Figure 2 shows amino acid acceptor activities of tRNAs of skeletal muscle from the three different ages when tested with eleven amino acids.

In general, there was a quantitative variation in the different aminoacyl-tRNAs at each age. Tyrosyl, arginyl; lysyl, alanyl, aspartyl-tRNA were in higher concentrations, whereas leucyl and prolyl-tRNAs were present in quite low amounts. The relative abundancy of these aminoacyl-tRNAs in muscle may be due to the preferential synthesis of muscle proteins. A number of studies showed that the tRNA complement of some tissues, specialized for the production of one or a few proteins, reflected the amino acid composition of that protein (Garel *et al.*, 1970, 1971; Chavancy *et al.*, 1971; Elska *et al.*, 1971; Smith and McNamara, 1972; Lanks and Weinstein, 1970; Maepaa and Ahonen, 1972). The proteins of the muscle, i.e.,



**Figure 1.** Transfer RNA from young, adult and old rat muscle was aminoacylated using aminoacyl-tRNA synthetases from young, adult and old rat muscle respectively.  $[^{14}\text{C}]$ -Algal protein hydrolysate was used as a source of amino acids.

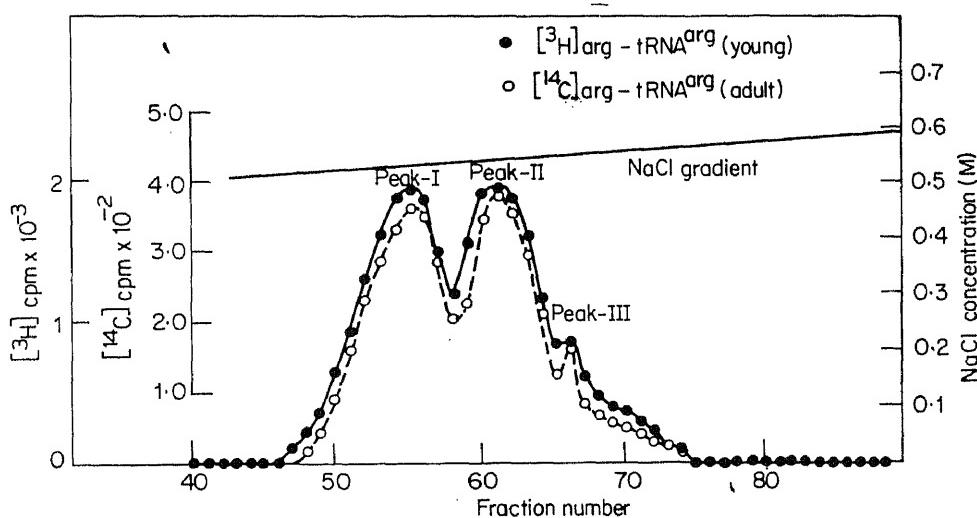


**Figure 2.** Transfer RNAs from skeletal muscle of young, adult and old rats were assayed using homologous aminoacyl-tRNA synthetases. Specific activities of  $[^{14}\text{C}]$ -amino acids used were as follows:

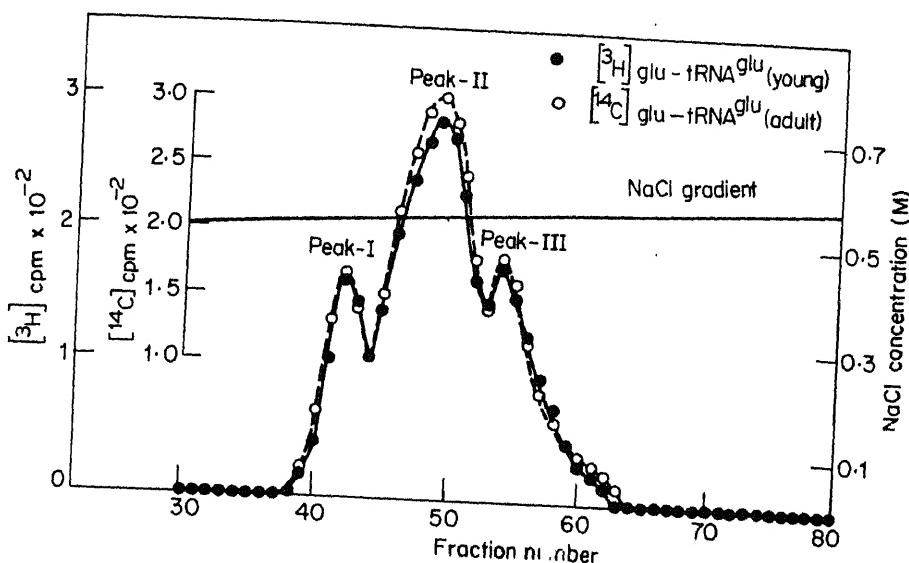
Tyr 40 mCi/mmol; Arg 150 mCi/mmol; Gly 50 mCi/mmol; Ser 75 mCi/mmol;  
Ala 75 mCi/mmol; Asp 100 mCi/mmol; Gly 125 mCi/mmol;  
Pro 125 mCi/mmol; Leu 150 mCi/mmol; Lys 150 mCi/mmol; Met 54 mCi/mmol.

myosin, actin, troponin and tropomyosin of rabbit were reported to be rich in glutamic acid, aspartic acid, leucine and lysine (Laki, 1971; Bodwell, 1971). Contrary to rabbit muscle proteins, the relative proportion of leucine in rat muscle proteins must be very low since leucyl-tRNA concentration was found to be very low in rat muscle.

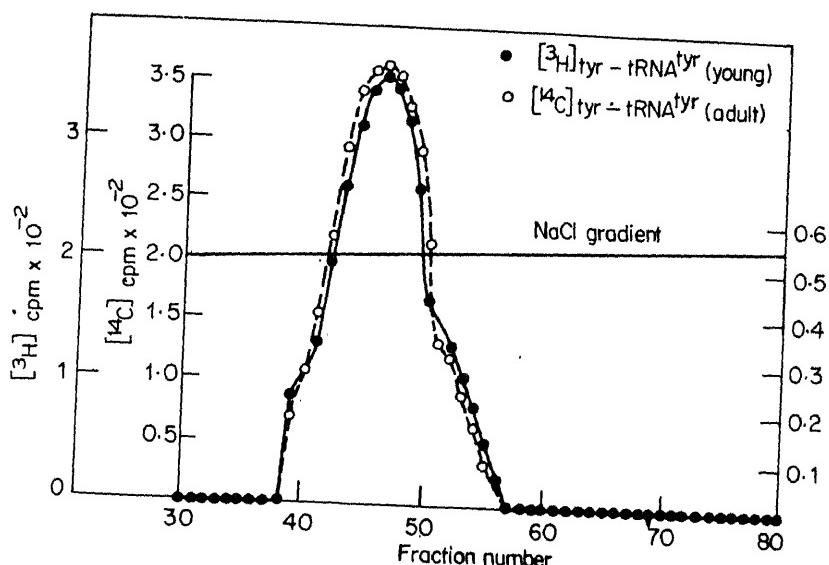
Fractionation of arginyl, glutamyl and tyrosyl-tRNAs are shown in figures 3, 4 and 5. Though arginyl and glutamyl-tRNAs fractionated into different isoacceptor species neither qualitative nor quantitative changes were found in the distribution of iso-acceptor species with age. Tyrosyl-tRNA did not fractionate under the experimental conditions used. The elution profile showed two shoulders which may represent two more isoacceptors. No major changes were found in the tyrosyl-tRNA elution profile as a function of age.



**Figure 3.**  $[^{14}\text{C}]$ -Arginine (354 mCi/mmol) and  $[^3\text{H}]$ -arginine (8.5 Ci/mmol) were used. In figure 3,  $[^3\text{H}]$ -arginyl-tRNA from young rat was mixed with  $[^{14}\text{C}]$ -arginyl-tRNA from adult and co-chromatographed. The column was developed as mentioned in material and methods. A similar fractionation pattern was observed when  $[^3\text{H}]$ -arginyl-tRNA from young rat was mixed and co-chromatographed with  $[^{14}\text{C}]$ -arginyl-tRNA from old. Percentage recovery was 85-95%. Peaks, I, II and III contain about 50%, 40% and 10% of total  $\text{arg-tRNA}^{\text{arg}}$  respectively in all the three ages.



**Figure 4.**  $[^3\text{H}]$ -Glutamic acid (1.77 Ci/mmol) and  $[^{14}\text{C}]$ -glutamic acid (284 mCi/mmol) were used.  $[^3\text{H}]$ -Glutamyl tRNA from young was mixed with  $[^{14}\text{C}]$ -glutamyl tRNA from adult in figure 4 and co-chromatographed. The column was developed as mentioned in materials and methods. Glutamyl-tRNA from old showed a similar pattern of fractionation of isoacceptor species. The precentage recovery was 78-96%. Peaks I, II and III contain about 20%, 60% and 20% of glu-tRNA<sub>glu</sub> respectively in all the three ages.



**Figure 5.**  $[^3\text{H}]$ -Tyrosine (2 Ci/mmol) and  $[^{14}\text{C}]$ -tyrosine (518 mCi/mmol) were used.  $[^3\text{H}]$ -tyrosyl-tRNA from young and  $[^{14}\text{C}]$ -tyrosyl-tRNA from adult was mixed for chromatography. The column was developed as mentioned in materials and methods. Percentage recovery was 90-98%. Tyrosyl-tRNA from old rat tissue showed a similar fractionation pattern.

## Discussion

Total tRNA content was maximum in the skeletal muscle of the young rats. Skeletal muscle is a post-mitotic tissue and its development continues upto about 50 days. The concentration of transfer RNA was maximum and it was also maximally active during developmental phases of the tissue, after which it showed a gradual decline with age.

The isoacceptor species of three aminoacyl-tRNAs did not show any qualitative or quantitative changes. Skeletal muscle, being a post-mitotic tissue, may not need any drastic changes in the isoacceptor species of tRNAs since there may not be too many different types of messengers appearing with age. All the isoacceptor species of these aminoacyl-tRNAs may be used for protein synthesis in young, adult and old rat muscle. The other possibility is that arginyl, glutamyl and tyrosyl-tRNAs may not be involved in regulation brought about by transfer RNA in general. In other words these aminoacyl-tRNAs may not be the modulating tRNA species. There may be other aminoacyl-tRNAs present in skeletal muscle of rats which act as modulating species of tRNA thereby regulating protein synthesis and consequently development and ageing. Whether any qualitative or quantitative changes are present in other aminoacyl-tRNAs of skeletal muscle remains to be studied.

## Acknowledgement

The investigation was supported by University Grants Commission, New Delhi.

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## Bacteriophage burst size during multiple infections

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MS received 11 March 1980; revised 14 August 1980.

**Abstract.** A significant positive correlation was observed between multiplicity of infection and burst size of mycobacteriophage I3. During multiple infections, the average contribution of each infecting phage to the burst size was inversely correlated with multiplicity of infection even when bacterial resources were not limiting. We conclude that the efficiency of phage-coded functions rather than the extent of bacterial resources determines the burst size.

**Keywords.** Bacteriophage; mycobacteriophage I3; burst size; multiple infections.

### Introduction

The last few decades have witnessed phenomenal advances in our understanding of the detailed mechanism of phage growth and reproduction (Lewin, 1977). Bacteriophages inject their DNA or RNA into a sensitive host bacterium and utilize the bacterial machinery to produce a number of progeny phage particles. The number of phages liberated by an infected bacterium at the end of one cycle of phage growth designated as the burst size is characteristic of a given phage-host system. Questions such as why a particular phage should have a certain burst size or to what extent the phages utilize the nutritional resources in the bacterium are still unanswered.

When a phage infects a bacterium, several phage and host factors come into play to produce a crop of phage particles. The actual number of phage particles produced may be determined by: (a) nutritional status of the bacterium if the phage utilizes the bacterial machinery maximally for its reproduction or (b) phage-coded functions such as polymerases and regulatory proteins essential for phage production; their inherently low efficiency may not permit the phage to make full use of bacterial resources and consequently the burst size may be limited. In this paper we describe results of experiments designed to distinguish between alternatives (a) and (b) and thus elucidate the factors which might be responsible for the burst size of a bacteriophage.

### Materials and methods

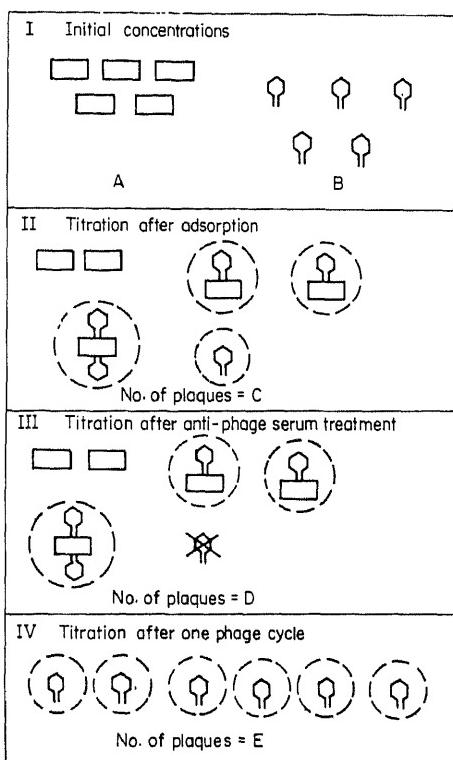
#### Organisms

*Mycobacterium smegmatis* SN2 and a clear plaque mutant (Gopinathan *et al.*, 1978) of its phage I3 (Sunder Raj and Ramakrishnan, 1970) were used.

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### Methods

The experiment consisted of four parts (figure 1): I. Desired numbers of bacteria (*A*) and phage (*B*) were mixed in nutrient broth. The number of bacteria in each ex-



**Figure 1.** Schematic representation of the experimental protocol  
Bacteria and phage are shown schematically. Each circled entity gives rise to one plaque when titrated with indicator bacteria.

periment was  $10^7$ . The number of phages was varied from experiment to experiment. II. At the end of 30 min of adsorption at  $37^\circ\text{C}$  in the presence of 3 mM KCN to synchronize infection and thus avoid any interference (Hutchison and Sinsheimer, 1966), the phage-bacteria mixture was titrated with indicator bacteria. Let the number of plaques obtained be *C* which equals the number of infected bacteria plus the number of free phages remaining. Each infected bacterium gives rise to only one plaque irrespective of the number of phages infecting it. III. Free phages were inactivated by the addition of antiphage serum and the titration was repeated. Let the number of plaques obtained now be *D* which equals the number of infected bacteria. IV. The mixture was diluted  $10^3$ -fold and incubated in a rotary shaker for one cycle of phage growth (4 h) (Gadagkar, 1979), lysed by the addition of chloroform and titrated again with indicator bacteria. Let the number of plaques obtained now be *E* which equals the total number of phages liberated at the end of one cycle

of growth. Then,  $B/A$ =multiplicity of infection defined as the ratio of phage:bacteria added;  $C-D$ =number of unadsorbed free phage;  $B-(C-D)$ =number of phages adsorbed;

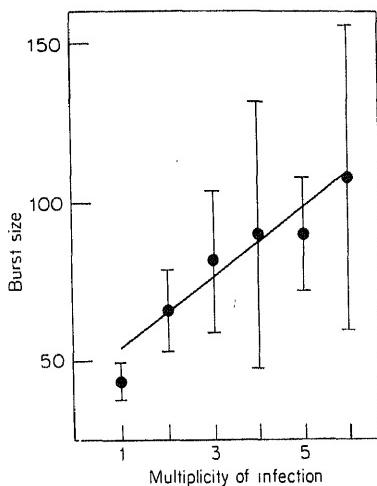
$$\frac{B-(C-D)}{D} = \text{mean number of phages adsorbed per infected bacterium,}$$

called the *effective multiplicity of infection*;  $E/D$ =burst size, defined as the mean number of phages liberated per infected bacterium and  $E/[B-(C-D)]$ =mean number of phages liberated per phage adsorbed, called the *effective burst size*. The experiment at each multiplicity of infection from 1-6 was repeated four to six times. The data were statistically analyzed by the student's *t* test and the level of significance is indicated in each case (Bailey, 1959).

## Results

### *Correlation between multiplicity of infection and burst size*

It can be seen from figure 2 that there was a significant positive correlation between multiplicity of infection and the burst size. This can be interpreted to mean that at low multiplicity of infection, the phages did not utilize the entire resources available in the bacterium and hence the burst size was limited by the inherent capacity of the

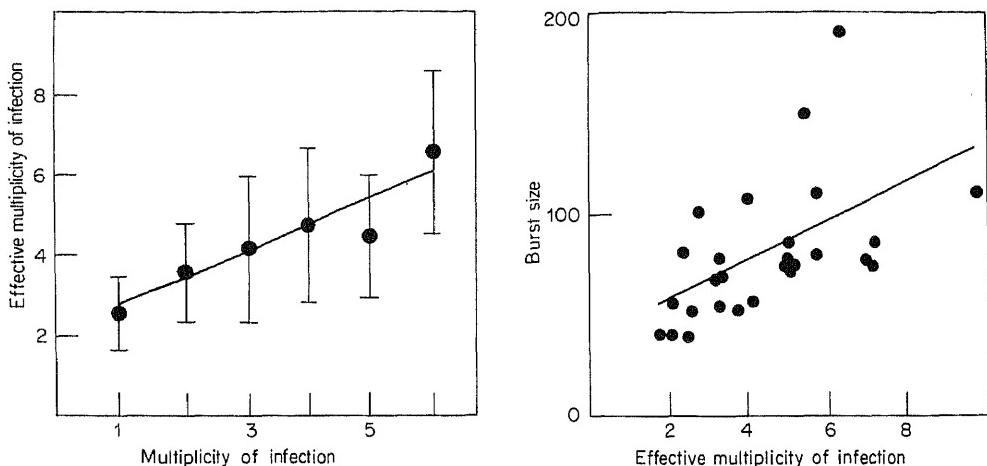


**Figure 2.** Correlation between multiplicity of infection and burst size  
Mean  $\pm$ S.D. from 4-6 experiments for each point are shown. The solid line is the least squares fit. Correlation coefficient = +0.58, significant at  $P \leq 0.01$ . Multiplicity of infection is defined as the ratio of phage: bacteria added and burst size is defined as the mean number of phages liberated per infected bacterium.

phage for reproduction. This does not imply that the nutritional status of the bacterium had no effect on the burst size but simply that under the given conditions, the phage did not make full use of the resources in the bacterium. In fact, the burst size is further reduced if the bacteria were growing in a synthetic minimal medium instead of nutrient broth (Gadagkar, 1979).

### *Correlation between multiplicity of infection and effective multiplicity of infection*

As the multiplicity of infection was increased, the effective multiplicity of infection (defined as the mean number of phages adsorbed per infected bacterium) also increased significantly (figure 3). At any given multiplicity, the effective multiplicity of infection was often higher than the initial value itself.



**Figure 3.** Correlation between multiplicity of infection and effective multiplicity of infection. Mean  $\pm$ S.D. from 4-6 experiments for each point are shown. The solid line is the least squares fit. Correlation coefficient = +0.62, significant at  $P < 0.001$ . Effective multiplicity of infection is defined as the number of phages adsorbed per infected bacterium.

**Figure 4.** Scatter diagram showing correlation between effective multiplicity of infection and burst size.

The solid line is the least squares fit. Correlation coefficient = +0.56, significant at  $P < 0.01$ .

### *Correlation between effective multiplicity of infection and burst size*

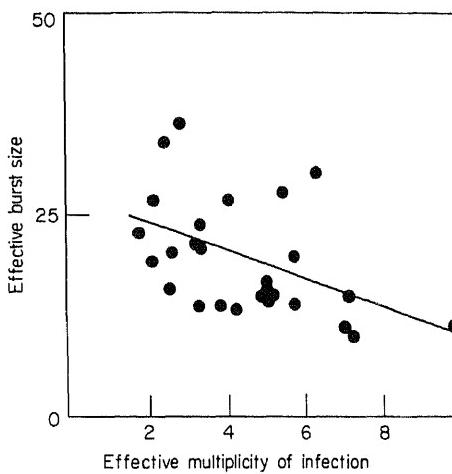
In spite of the fact that effective multiplicity of infection was often slightly higher than multiplicity of infection, there was a significant positive correlation between effective multiplicity of infection and burst size (figure 4). This result confirms that as the number of phages infecting a bacterium increased, phage production also increased.

### *Correlation between effective multiplicity of infection and effective burst size*

Since the burst size increased during multiple synchronous infections (the phrase 'multiple infection' is used throughout to represent the simultaneous infection of a bacterium by more than one phage), it was interesting to see how the phages interacted with one another and contributed to the total phage production. For this reason we have computed the effective burst sizes (mean number of phages liberated

per phage adsorbed). Figure 5 shows that there was a significant negative correlation between the effective multiplicity of infection and effective burst size. Therefore, during the multiple infections, the contribution of each phage to the total burst size decreased with increasing number of phages infecting a bacterium. The negative correlation between effective multiplicity of infection and effective burst size does not mean that the bacterial machinery becomes limiting because the burst size is positively correlated with effective multiplicity of infection. This suggested that although the bacterial machinery was not limiting, there was some kind of interaction among the phages during multiple infection which reduced the effective burst size.

It must be pointed out that despite the apparent scatter, all the correlations are highly significant as indicated by students' *t* test ( $P < 0.01$ ).



**Figure 5.** Scatter diagram showing correlation between effective multiplicity of infection and effective burst size

The solid line is the least squares fit. Correlation coefficient =  $-0.49$ , significant at  $P < 0.01$ . Effective burst size is defined as the mean number of phages liberated per phage adsorbed.

## Discussion

Delbrück and Luria (1942) working with phages  $\alpha$  and  $\beta$  infecting *E. coli* observed that the burst size during multiple infections was higher than during single infections but that there was no correlation between multiplicity of infection and burst size. In contrast, it is known in a number of systems that if lysis of the infected bacterium is delayed, there is an increase in the burst size. This has been achieved in the case of T-even  $r^+$  phages by superinfection within a few minutes of the primary infection (Doerman, 1948) or by the inhibition of protein synthesis in the middle of the latent period (Hershey and Melechen, 1957) and in the case of  $\phi X174$  by infection with a lysozyme defective mutant (Hutchison and Sinsheimer, 1966). Doerman (1948) has made a detailed study of the mechanism of lysis inhibition by T-even phages. Although not explicitly mentioned, it is clear from his data that even at a multiplicity of infection which did not bring about an extension of the latent period by delaying lysis, there was a positive correlation between multiplicity of infection and burst size.

Hutchinson and Sinsheimer (1966) working with a lysis defective mutant of the phage  $\phi$ X174 have shown that maturation of phages continued longer than in the case of wild type infection leading to a 10-fold increase in the burst size. This suggests that in wild type infections, maturation of phage particles within a bacterium is not a synchronous process and that lysozyme is produced much before all the potential progeny mature. It may be reasonable to conclude that multiple infections result in a situation similar to that of lysis inhibition and the increase in burst size observed under these conditions may be due to similar mechanisms. For example, if maturation of phages is controlled by limiting concentrations of one or more phage-coded catalytic factors, multiple infections can lead to increased phage production before the onset of lysis even in wild type conditions. This is because there would be an increased supply of those limiting factors. However, during multiple infections, the threshold concentrations of lysozyme needed to lyse the cell also might be reached somewhat earlier so that each phage produces less progeny than in single infections and yet the total phage production in one bacterium can be higher than in single infections.

Thus it could be a general property of phages that they utilize a small proportion of the bacterial resources in the course of their reproductive cycle. While the reasons for this are not obvious, it suggests that the efficiency of phage-coded regulatory factors involved in phage development and reproduction determine the burst size. That the burst size can still be reduced by decreasing the nutritional content of the bacteria (Gadagkar, 1979) suggests that phages have evolved to utilize only a small proportion of whatever resources are present in the bacterium. One possible reason for this is that in nature it may be very important for the phage to lyse the bacterium as quickly as possible. This may be because of a certain finite probability of bacterial death which would increase with time. However, going through more generations rather than utilizing all the resources available in one bacterium may help leave more progeny behind. Thus the burst size may be adapted to adjust to such factors as availability of hosts, probability of death of hosts and the time and energy required to channel the host towards phage production. This would explain why phages could have evolved to utilize only a small proportion of whatever resources are available in a bacterium and also why maturation of the progeny is asynchronous leaving the actual time of lysis as the flexible control point.

The present study indicates that more detailed investigations on the molecular mechanisms involved in resource utilization by bacteriophages are needed. Similar experiments could then be extended to pairs of different mutants of a bacteriophage or even to different bacteriophage species with a common host. This should lead to an understanding of the molecular basis of ecological isolation (Hardin, 1960), that is, those subtle differences in the requirements of different bacteriophages with a common host, that have made it possible for them to coexist.

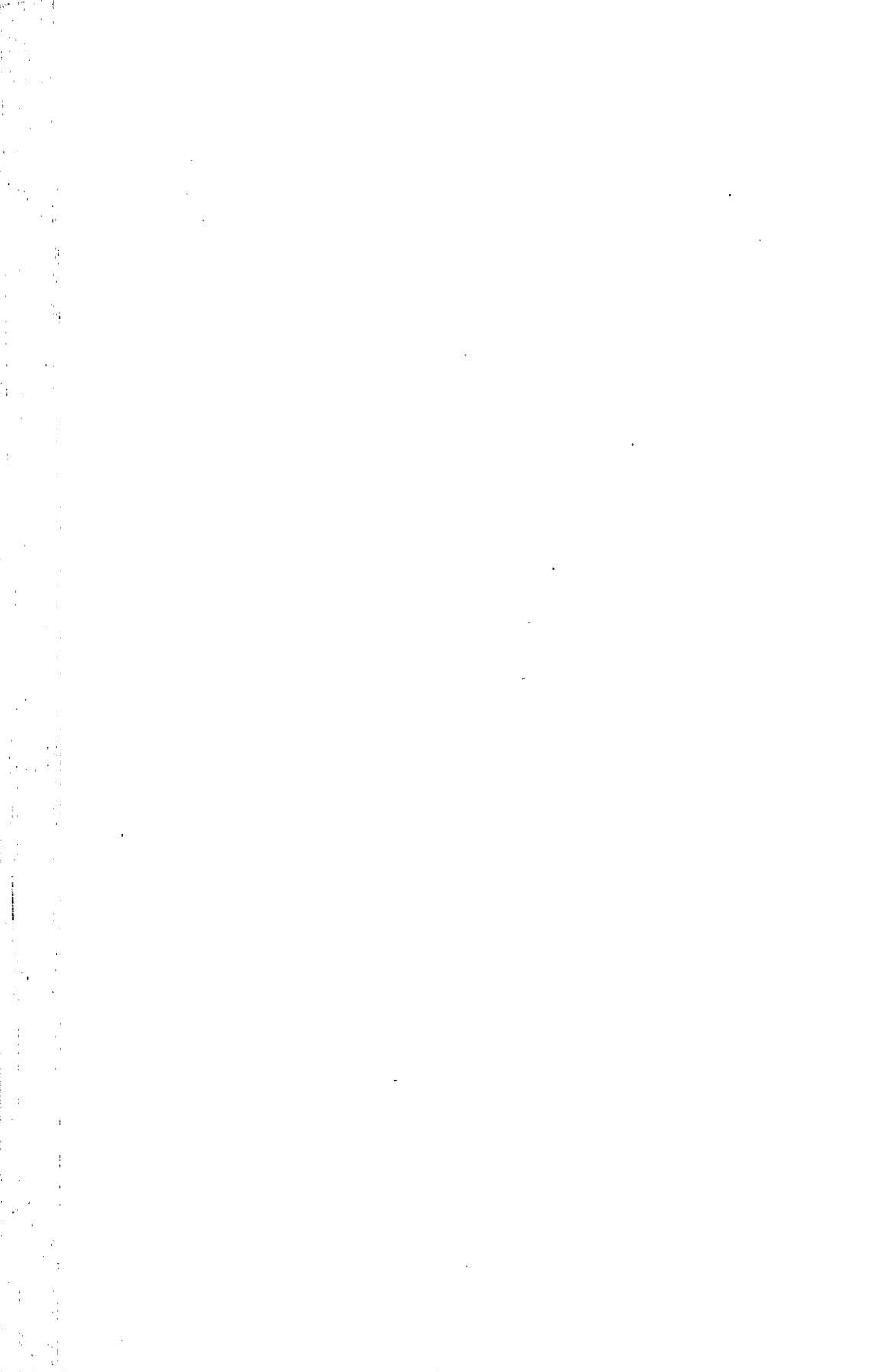
### Acknowledgements

We are grateful to Drs V. Nanjundaiah, Madhav Gadgil, H. Sharat Chandra and N. V. Joshi for helpful discussions.

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## **Ultrastructural studies on the epididymal spermatozoa in the rhesus monkey**

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MS received 26 May 1980; revised 6 August 1980

**Abstract.** Ultrastructural studies on the spermatozoa in different regions of the epididymis of the rhesus monkey have shown that the process of sperm maturation is associated with the caudad migration of the cytoplasmic droplet, a reduction in the volume of the cytoplasmic droplet, and an obvious wrinkling of the plasma membrane surrounding the head of the spermatozoa. These changes are completed by the time the spermatozoa reach the distal-middle segment of the epididymis. The present studies also indicate that spermatozoa are incorporated into the intraepithelial cells in the epididymis. This finding suggests that spermiophagy is a normal occurrence in the epididymis of rhesus monkey.

**Keywords.** Ultrastructure; epididymis; spermatozoa; rhesus monkey.

### **Introduction**

The epididymis has been recognized as a potential site for the extraneous intervention for purpose of regulating fertility. This conclusion is based on the knowledge that spermatozoa undergo a process of maturation during their passage through the epididymis.

The process of sperm-maturation can be microscopically visualized by the location of the cytoplasmic droplet on the spermatozoa. The cytoplasmic droplet is located in the neck region in such of the spermatozoa which have not yet undergone maturation, whereas in the matured spermatozoa cytoplasmic droplet is either located at the distal end of the midpiece (Bloom and Nicander, 1961) or it is shed-off (Bedford *et al.*, 1973) depending on the species.

A detailed light microscopic study of the epididymis of the rhesus monkey has revealed that marked histological differences occur in the histological features of the epithelium in the different regions of the epididymis (Prakash *et al.*, 1979). These structural differences have been assumed to reflect functional differences between the epididymal segments and appear to be related to the maturation of spermatozoa. In the studies reported here, we have sought to determine the topographic region of the epididymis where spermatozoa mature, as evidenced by

the location of the cytoplasmic droplet on the spermatozoa. This information is of relevance to the evaluation of anti-fertility agents affecting sperm-maturation in the epididymis of non-human primate species.

In the course of these studies we have also observed, for the first time, that spermatozoa are resorbed in the epididymis of the rhesus monkey under normal physiological conditions; since the process of sperm-resorption may be related to the production of sperm-antibody, this interesting feature is also reported.

## **Materials and methods**

Tissues were obtained from the different epididymal segments (figure 1) of eight, healthy adult rhesus monkeys. The demarcation of the epididymis into initial, middle and terminal segments respectively, was based on histological criteria (Prakash *et al.*, 1979). The tissues were fixed by vascular perfusion (Anand Kumar *et al.*, 1980a) and processed for electron microscopy (Anand Kumar *et al.*, 1980b).

## **Results**

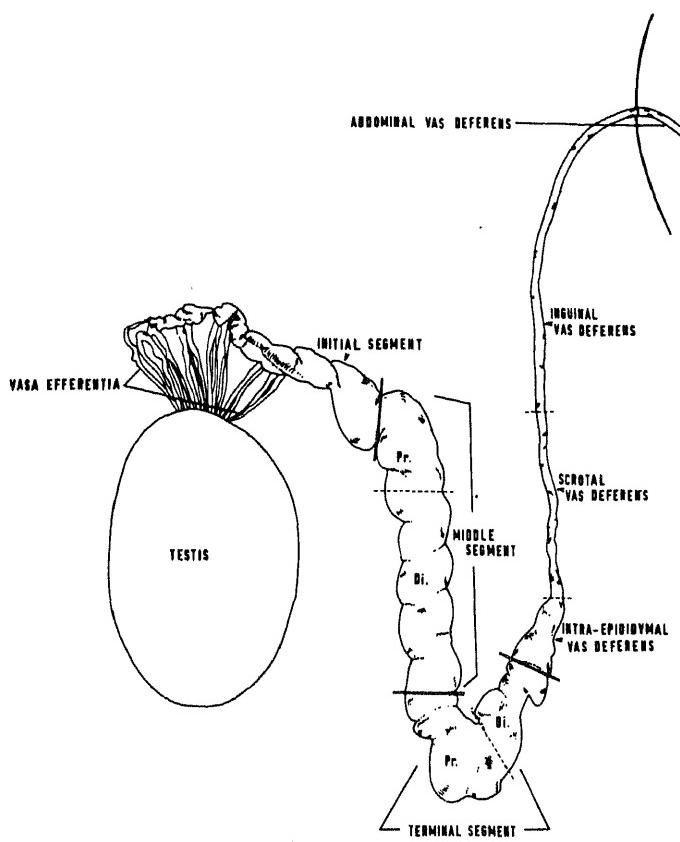
### *Sperm maturation*

The cytoplasmic droplet occurs as a cuff of residual cytoplasm surrounding the post-acrosomal portion of the spermatozoa. In spermatozoa present in the initial segment of the epididymis, the cytoplasmic droplet is attached to the proximal portion of the midpiece of the spermatozoa (figure 2). The plasma membrane in most of the spermatozoa in the initial segment lies in close apposition to the acrosomal membrane.

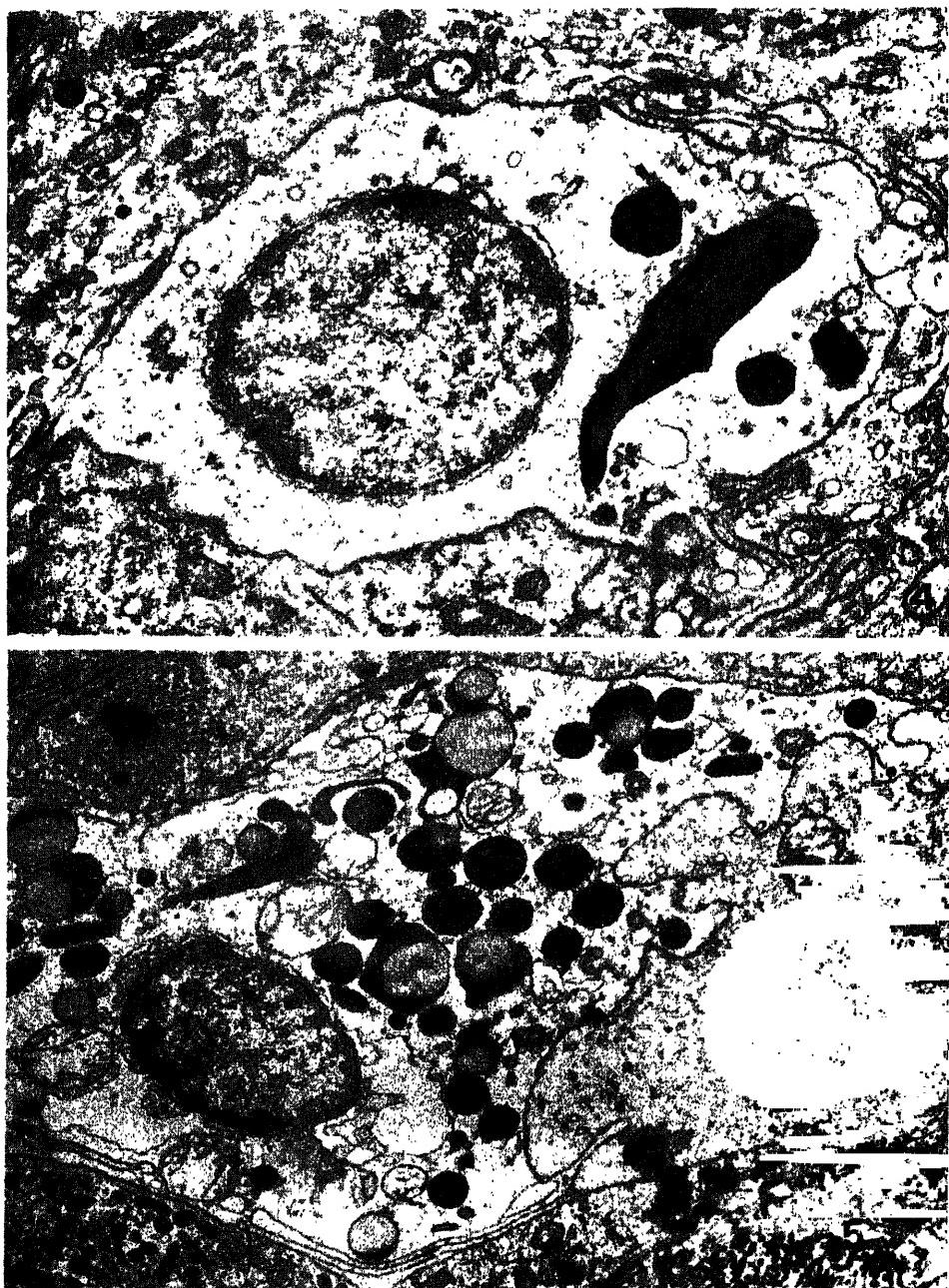
Some of the spermatozoa from the proximal-middle segment (figure 1) of the epididymis show a translocation of the cytoplasmic droplet to the distal end of the midpiece of the spermatozoa. In the distal-middle (figure 1) as well as in the terminal segments of the epididymis, the cytoplasmic droplet, in most of the spermatozoa, is located in the distal part of the midpiece (figure 3). Coincident with this translocation, the volume of the cytoplasmic droplet decreases and the plasma membrane of the spermatozoa in the head region is wrinkled and separated off from the acrosomal membrane.

### *Spermiophagy*

Remnants of spermatozoa occur within the cytoplasm of intraepithelial cells. These cells are pleomorphic with an euchromatic nucleus and they constitute a wandering population of cells. The other cytoplasmic inclusions of these cells comprise lysosomes, lipofuscin granules and lipid droplets (figures 4 and 5) which suggests that they may be similar to the 'macrophage-like' basal cells described in previous studies (Ramos and Dym, 1977; Prakash *et al.*, 1979).



**Figures 1-3.** 1. Diagrammatic representation of the testis, the vasa efferentia, the epididymis and the vas deferens. The different regions of the epididymis taken for ultrastructural studies are indicated in the diagram. Pr: Proximal. Di: Distal. 2. Ultrastructure of a spermatozoon from the initial segment of the epididymis. Note that the cytoplasmic droplet surrounds the neck of the spermatozoon. X9000. 3. Ultrastructure of a spermatozoon from the terminal segment of the epididymis. The cytoplasmic droplet is translocated to the caudal part of the spermatozoon. X9000.



**Figures 4-5.** A part of a spermatozoon's head can be seen within an intraepithelial cell in the epididymal epithelium. X10,000. 5. Electron micrograph illustrating two adjacently lying intraepithelial cells in the epididymal epithelium. A part of a spermatozoon and cytoplasmic inclusions comprising lipofuscin granules and lysosomes can be seen in one of the intraepithelial cells. X10,000.

## Discussion

A number of criteria such as, acrosomal changes, motility pattern of spermatozoa and changes in the biochemical constituents of spermatozoa have been used by different investigators to identify events related to the maturation of spermatozoa (Orgebin-Crist *et al.*, 1975; Bedford 1975). Microscopic changes reported in relation to the maturation of spermatozoa are the caudad migration of the cytoplasmic droplet and, coincident with this migration, there is a reduction in the volume of the cytoplasmic droplet and a wrinkling of the plasma membrane surrounding the head of the spermatozoa (Bloom and Nicander, 1961; Fawcett and Philips, 1969; Zamboni *et al.*, 1971).

In rodents (Bedford 1975), the fully matured spermatozoa in the terminal segment of the epididymis lack cytoplasmic droplets since these are sloughed off. Alsum and Hunter (1978) came to a similar conclusion in the rhesus monkey, on the basis of their optical microscopic studies on spermatozoa in the cauda epididymidis. The present ultrastructural studies, however, indicate that the vast majority of spermatozoa in the terminal segment of the epididymis do not entirely shed off their cytoplasmic droplets—a situation that is similar to that described in the human epididymis (Bedford *et al.*, 1973). The reasons for the difference between our results and those of Alsum and Hunter (1978) are not clear but may be due to differences in the microscopic methods used for observation.

The present studies have also shown that the ultrastructural changes attributed to the maturation of spermatozoa are first noticed in the proximal-middle segment of the epididymis where maximal fluid-resorption occurs (unpublished observations). It could well be that ionic shifts in the luminal fluid, resulting from fluid resorption (Crabo, 1965; Levin and Marsh, 1971) contribute to the caudad migration of the cytoplasmic droplet as has been suggested previously (Bedford, 1975). If this is so, then one can conclude that the maturation of spermatozoa in the rhesus monkey is almost completed by the time spermatozoa reach the distal middle segment of the epididymis.

The present observations have revealed that parts of spermatozoa occasionally occur within the cytoplasm of the wandering population of intraepithelial cells in the epididymis of intact, untreated adult male rhesus monkeys. The intraepithelial cells have features characteristic of a phagocytic function, *viz.*, presence of lysosomes and lipofuscin granules which have been shown to stain meta-chromatically with toluidine blue (Prakash *et al.*, 1979). The presence of spermatozoa within these cells suggests that these cells are capable of spermophagy, in keeping with an earlier suggestion (Prakash *et al.*, 1980). The question as to how the spermatozoa traverse the apically situated tight occluding junctions between the adjoining epithelial cells (Anand Kumar *et al.*, 1980a) needs to be elucidated as also the functional significance of spermophagy under normal physiological conditions.

## Acknowledgements

This work was supported by the Indian National Science Academy. Technical assistance of Shri S. C. P. Sharma is gratefully acknowledged.

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## Arginase from rat fibrosarcoma. Purification and properties

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**Abstract.** Arginase from rat fibrosarcoma was purified about 1900-fold and its properties were compared with those of the enzyme from liver and kidney. Arginase from fibrosarcoma was a neutral protein of molecular weight 120,000 with a  $K_m$  value of 11 mM for arginine. The activation energy was 7.2 kcal/mol and the pH optimum was 10. The fibrosarcoma enzyme was immunologically different from that of the liver. The arginase from fibrosarcoma closely resembled the arginase from the kidney in its electrophoretic, kinetic and immunological properties.

**Keywords.** Rat fibrosarcoma; arginase; immune-precipitation; urea cycle.

### Introduction

Arginase (L-arginine amidinohydrolase, EC 3.5.3.1) is present in many rat tissues with the highest activity present in liver (Greengard *et al.*, 1970). Among the extrahepatic tissues, sub-maxillary salivary glands possess the highest arginase activity (Herzfeld and Raper, 1976). Arginases from rat liver, kidney, lactating mammary gland, small intestine and submaxillary gland were purified and their properties were studied (Schimke, 1964; Kaysen and Strecker, 1973; Glass and Knox, 1973; Fujimoto *et al.*, 1976; Gopalakrishna and Nagarajan, 1980 b).

Unlike in tumours of the submaxillary gland, high arginase activity was observed in viral, chemically-induced and also in spontaneous mammary gland tumours (Rogers and Moore, 1971; North *et al.*, 1971; Kesava Rao *et al.*, 1976; Bhide, 1971; Herzfeld and Raper, 1976). The properties of arginases from rabbit skin papillomas, hamster fibrosarcoma and mouse lung adeno-carcinoma have been studied (North *et al.*, 1971; Orth *et al.*, 1967; Kesava Rao *et al.*, 1978). We have attempted to purify arginase from rat fibrosarcoma and compared it with the enzyme from normal tissues like liver and kidney.

### Materials and methods

#### Chemicals

DEAE-cellulose (DEAE-11) and CM-cellulose (CM-11) were purchased from Whatman Ltd., Maidstone, Kent, UK; arginine and diacetylmonoxime from Sigma Chemical Co., St. Louis, Missouri, USA; Sephadex G-100 from Pharmacia Fine Chemicals, Uppsala, Sweden; Freund's complete adjuvant from Difco Lab., Detroit, Michigan, USA.

The fibrosarcoma was induced in rats by 2-methyl-cholanthrene and propagated in rats by serial transplantation (Nagarajan and Sankaran, 1973).

### *Assay for arginase activity*

The enzyme activity was measured as described previously (Gopalakrishna and Nagarajan, 1979a). One ml of reaction mixture contained 40 mM glycine-NaOH buffer (pH 9.5) and 150 mM arginine (pH 9.5). After incubation at 37°C for 10 min, the reaction was stopped by adding 2 ml of 10% trichloroacetic acid. The urea formed was estimated by using diacetylmonoxime. Since sucrose interferes with urea estimation, for samples in sucrose medium ornithine formed was estimated using the modified Chinard's ninhydrin method (Gopalakrishna and Nagarajan, 1980a). One unit of enzyme is the amount that catalyses the production of 1 µmol of urea/min.

### *Protein estimation*

Protein was estimated by the method of Lowry *et al.*, (1951) using bovine serum albumin as the standard. Manganese present in enzyme preparations interfered with the colour development and was removed as insoluble manganese phosphate; to 0.5 ml of protein in solution, 0.5 ml of 0.2 M sodium phosphate buffer (pH 8.0) was added and after keeping 10 min at room temperature, the mixture was centrifuged at 600 g for 5 min. The supernatant was used for protein estimation.

### *Purification procedure*

The steps used for the purification of arginase from liver were adapted from the procedures of Schimke (1964) and Tarrab *et al.*, (1974). For the isolation of kidney arginase, purification steps upto the ammonium sulphate precipitation were identical to that used by Kaysen and Strecker (1973). The purification procedure of arginase from fibrosarcoma closely resembled the procedure for the kidney enzyme.

Unless otherwise stated, all the steps of purification were carried out at 6°C and centrifugations were done at 13,000 g at 0°C for 15 min.

Fibrosarcoma tissue weighing 50 g was homogenized in 4 volumes of 0.01 M; Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and centrifuged at 600 g for 15 min. The supernatant was further centrifuged at 13,000 g for 15 min. The sediment was suspended in 20 ml of 0.01 M Tris-HCl/0.01 M MnCl<sub>2</sub>/0.005 M arginine (pH 7.4) and subjected to freezing and thawing. After centrifugation the clear supernatant containing arginase activity was subjected to ammonium sulphate precipitation.

### *Ammonium sulphate precipitation*

The amount of ammonium sulphate required to obtain 50% saturation was added with constant stirring, maintaining the pH at 7.4 by the addition of 1 M Tris base. The mixture was kept in ice for 30 min and centrifuged. The precipitated protein was dissolved in 0.01 M Tris-HCl/0.005 M MnCl<sub>2</sub>/0.005 M arginine (pH 8.3) and dialyzed against the same buffer for 12 h with three changes of buffer.

### *Heat treatment*

The residue after dialysis was kept in a water bath at 60°C for 30 min and immediately cooled in ice for 10 min. The supernatant fraction containing arginase activity obtained on centrifugation was subjected to DEAE-cellulose chromatography.

### *DEAE-cellulose chromatography*

The enzyme was adsorbed on to a column of DEAE-cellulose (1.5×15 cm, OH<sup>-</sup> form) equilibrated with 0.01 M Tris-HCl buffer, pH 8.3 and the un-adsorbed proteins were washed away with the same buffer. The bound-proteins were eluted with a linear KCl gradient (0-500 mM) in 200 ml of 0.01 M Tris-HCl buffer, pH 7.4 at a flow rate of 40 ml/h. Fractions (4 ml) were collected. The fractions containing enzyme activity were pooled and dialyzed against 0.01 M Tris-HCl buffer (pH 7.4) and subjected to gel filtration.

### *Gel filtration*

The enzyme preparation after concentration to 2 ml with Aquacide was applied on to a column of Sephadex G-100 (2×50 cm) which was equilibrated with 0.01 M Tris-HCl buffer (pH 7.4) containing 0.1 M KCl and eluted with the same buffer. Fractions of 2 ml were collected.

As the enzyme after the gel filtration step was not stable to storage or concentration, further purification was not attempted.

### *Polyacrylamide gel electrophoresis*

The enzyme preparation, after the heat-treatment step, was subjected to the centrifuge-column technique to remove low-molecular-weight compounds (Gopalakrishna and Nagarajan, 1979b) and used for checking the electrophoretic mobility on polyacrylamide gel electrophoresis using 7.5% gels in 0.05 M Tris-glycine buffer (pH 8.6) (Davis, 1964). A current of 6 mA per tube was applied for 3 h. Arginase activity was determined in the latitudinal segments (0.3 cm thick) of the gel by immersing slices in the arginase assay mixture and incubating at 37°C for 30 min.

Kinetic studies were carried out using the enzyme obtained after DEAE-cellulose chromatography. The molecular weight was estimated by gel filtration (Andrews, 1964). Ribonuclease T<sub>1</sub> ( $M_r$ , 13,500), myoglobin ( $M_r$ , 17,500), cymotrypsinogen ( $M_r$ , 25,000), bovine serum albumin ( $M_r$ , 67,000), liver ornithine carbamoyl transferase ( $M_r$ , 105,000), bovine gamma globulin ( $M_r$ , 150,000) were used as standards.

Antiserum was raised to purified liver arginase in rabbits. The enzyme preparation of about 1 ml, containing 2 mg protein, was emulsified with 1 ml of Freund's complete adjuvant and injected into the hind limbs of the rabbits intramuscularly. After 10 days, a booster dose was given similarly. Two weeks later the animals were bled and the antiserum was stored at -10°C.

The immune-precipitation reaction was carried out in a 15-ml centrifuge tube. The reaction mixture, containing 40 µmol Tris-HCl buffer (pH 7.4), 10 to 15 µl antiserum and 0.2 ml of tissue homogenate containing 0.2 to 2 units of arginase activity in a final volume of 10 ml was incubated at 37°C for 3 h. Then the precipitated antigen-antibody complex was removed by centrifugation. Arginase activity was estimated in the supernatant.

## **Results and discussion**

The purification of arginase from fibrosarcoma is shown in table 1. During purification, the enrichment of arginase from fibrosarcoma resembled closely that of the kidney arginase but differed from that of the liver enzyme. In kidney and fibrosarcoma, the

enzyme was located in the mitochondria whereas the enzyme was present in the cytosolic fraction of the liver (Kaysen and Strecker, 1973; Rosenthal *et al.*, 1956).

**Table 1.** Purification of arginase from fibrosarcoma.

Step	Total protein (mg)	Total activity units	Specific activity units/mg	Yield %	Fold purification
Homogenate	14036	1560	0.11	100	1
Whole mitochondrial fraction	3453	1010	0.29	64	3
Mitochondrial extract	1218	958	0.78	61	7
Ammonium sulphate (50%)	396	870	2.19	55	20
Heat treatment	158	1695	10.7	108	97
DEAE-cellulose chromatography	25	1370	54.8	87	498
Ammonium sulphate (60%)	22	1180	53.6	75	487
Sephadex G-100 gel filtration	5	1065	213.0	68	1936

50 g of tumour was used each time. One unit of enzyme produces one  $\mu\text{mol}$  of urea per min.

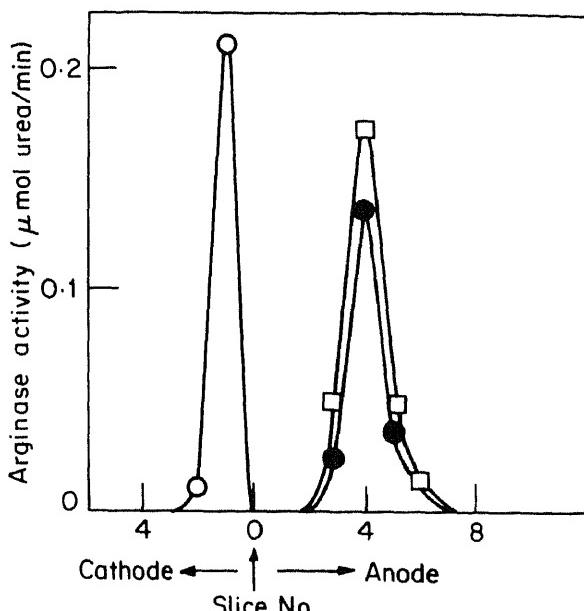
The liver arginase was stable at 60°C for 20 min, whereas the kidney enzyme was stable for 10 min. But by heat treatment, a two-fold increase in arginase activity of fibrosarcoma was observed. An increase in arginase activity upon heat treatment was also observed for the enzyme from bovine liver and mouse lung adenocarcinoma (Harell and Sokolovsky, 1972; Kesava Rao *et al.*, 1976). No attempt was made to identify the reason for the increase in the activity in the fibrosarcoma enzyme observed in this study.

The liver enzyme bound to CM-cellulose at pH 7.4, but the fibrosarcoma and kidney arginases were not adsorbed. None of these three enzymes bound to DEAE-cellulose at pH 7.4. But the fibrosarcoma and kidney arginases were bound to DEAE-cellulose at pH 8.3 while the liver arginase was not bound.

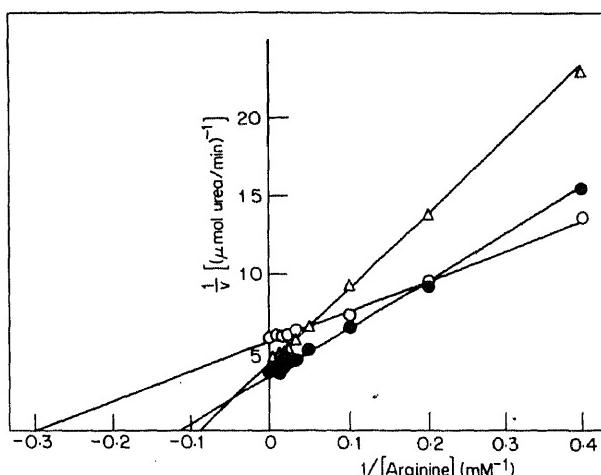
The liver arginase was quite stable during the purification and storage at 4°C and -10°C. But fibrosarcoma and kidney arginases were unstable after their release from mitochondria. During purification and storage, these were protected by the addition of arginine.

At pH 8.6, arginases from fibrosarcoma and kidney are anionic as seen by their electrophoretic mobility, whereas the liver enzyme is cationic (figure 1).

The  $K_m$  of arginase from fibrosarcoma for L-arginine was 11.0 mM (figure 2). But it was 3.3 mM and 9.0 mM for liver and kidney enzymes, respectively. The molecular



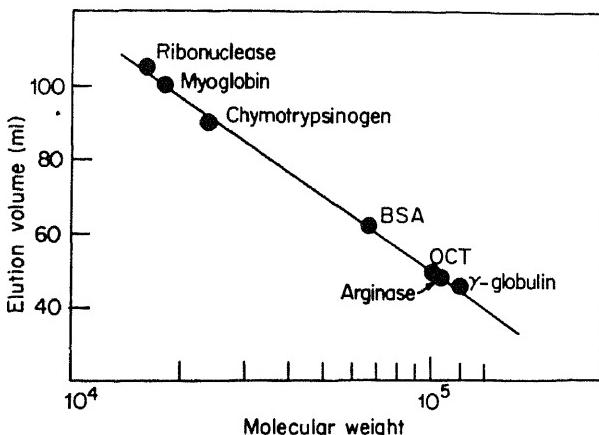
**Figure 1.** Polyacrylamide gel electrophoresis of arginases from fibrosarcoma, liver and kidney. After purification through ion-exchange cellulose chromatography step, arginase (2 units) from the three tissues were subjected to electrophoresis in 7.5% gels using 0.05 M Tris-glycine buffer (pH 8.6) for 3 h. Arginase activity was determined in 0.3 cm thick slices of the gel, fibrosarcoma (□); kidney (●); liver, (○).



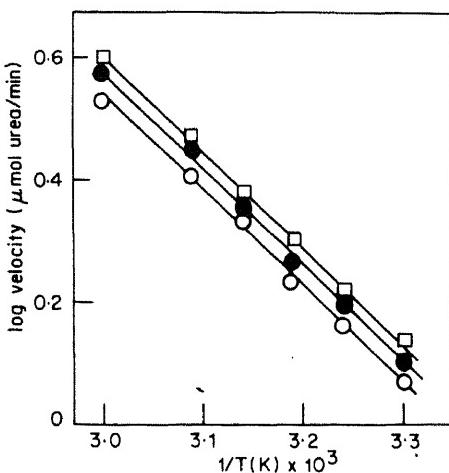
**Figure 2.** Lineweaver and Burk plots for the determination of  $K_m$  values of arginases for L-arginine

Fibrosarcoma, (□); liver, (○); kidney, (●).

weight of arginase from fibrosarcoma was found to be 120,000 (figure 3). The liver and kidney enzymes also have the same molecular weight. The activation energy for fibrosarcoma arginase was found to be 7.2 kcal/mol. It was 7.3 and 7.2 kcal/mol for the liver and kidney enzymes respectively (figure 4). The pH optimum for the reaction catalyzed by the arginase from fibrosarcoma was found to be 10. Liver and kidney enzymes showed pH optima of 10.5 and 10, respectively.



**Figure 3.** Estimation of molecular weight by Sephadex G-100 gel filtration. Protein (1-2 mg.) was applied in one ml to a column of Sephadex G-100 (2×50 cm) previously equilibrated with 0.01 M Tris-HCl buffer (pH 7.4) containing 0.1 M KCl and eluted with the same buffer with a flow rate of 20 ml/h. Fractions of 2 ml were collected and protein was estimated.



**Figure 4.** Arrhenius plots for the determination of activation energy. Arginase activity was estimated at various temperatures between 30°C and 60°C. Fibrosarcoma, (□); liver, (○); and kidney, (●).

The antiserum raised against liver arginase completely precipitated the enzyme from liver. But when the fibrosarcoma tissue extract was used, only 15-20% of the enzyme activity was precipitated (figure 5). Arginase from kidney was not precipitated by the antiserum to the liver enzyme. This observation shows that the arginases from kidney and fibrosarcoma are antigenically different from the liver enzyme. The 20% precipitation seen in fibrosarcoma tissue extract could be due to contamination by some liver-type arginase.

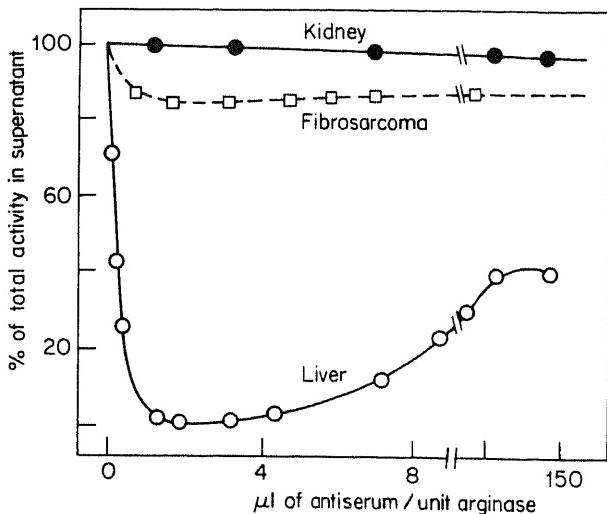


Figure 5. Immunoprecipitation of arginases from fibrosarcoma, kidney, and liver with antiserum to liver arginase.

The antiserum to liver arginase (10 to 50  $\mu$ l) was incubated with 0.2 ml tissue homogenate (0.2 to 2 units of arginase) at 37°C for 3 h; centrifuged and the supernatant was assayed for arginase activity.

Arginase is involved in urea synthesis in liver, where the urea cycle is operating. It is not clear what function this enzyme has in the kidney and fibrosarcoma where the urea cycle is not operative as indicated by the absence of ornithine carbamoyl transferase.

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## **Arginase from rat fibrosarcoma. Its possible role in proline, glutamate and polyamine metabolism**

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MS received 5 June 1980

**Abstract.** The presence of arginase in rat fibrosarcoma not synthesizing urea, suggested that this enzyme may have additional functions. Ornithine carbamoyl transferase, a key enzyme of the urea cycle was absent in this tissue, when compared to normal tissues, lower amount of ornithine was found in the fibrosarcoma, but this tumour contained a higher level of proline. The radioactivity present in L-[U-<sup>14</sup>C] arginine was incorporated into putrescine, spermidine, spermine, proline glutamate and glutamine suggesting that arginine was a possible precursor and that arginase may have a role in the synthesis of these metabolites.

**Keywords.** Rat fibrosarcoma; arginase; ornithine carbamoyl transferase; polyamines; proline; glutamate.

### **Introduction**

Arginine metabolism in tumours is still not clearly understood. Inhibition of tumourogenesis was achieved by feeding rats with a diet enriched with arginine (Takeda *et al.*, 1975). Arginase (L-arginine amidino hydrolase EC 3.5.3.1) activity was reported in the many non-hepatic tumours in which no other urea-cycle enzymes could be detected (Rogers and Moore, 1971; North *et al.*, 1971; Kesava Rao *et al.*, 1976; Herzfeld and Raper, 1976; Bhide, 1971). The relatively high arginase activity in the fibrosarcoma (Gopalakrishna and Nagarajan, 1980d), in the absence of the key urea cycle enzyme ornithine carbamoyl transferase (EC 2.1.3.3), suggested that arginase probably had other functions in addition to ureogenesis.

The diamine putrescine and the polyamines spermidine and spermine are present in all mammalian tissues including tumours. Ornithine decarboxylase (EC 4.1.1.17), a cytosolic enzyme converts ornithine to putrescine which is a precursor of spermidine. Ornithine carbamoyl transferase, a mitochondrial enzyme also utilizes ornithine for citrulline synthesis. Another mitochondrial enzyme ornithine transaminase (EC 2.6.1.13) catalyses the reversible formation of glutamic- γ -semialdehyde from ornithine. The free energy for this reaction favours the formation of glutamic- γ -semialdehyde (Strecker, 1965). The glutamic- γ -semialdehyde is then either reduced to proline or oxidized to glutamate. The synthesis of ornithine from proline and glutamate has also been shown in mammalian tissues (Smith *et al.*, 1967). Volpe and Strecker (1968) suggested an amphidirectional regulation of ornithine transaminase, and

postulated that in the liver, glutamate and proline serve as precursors for arginine, whereas the reverse situation is operative in the kidney. In order to examine this possibility, we estimated arginine and other related metabolites in fibrosarcoma and the rate of arginine catabolism using radiolabelled arginine.

## Materials and methods

### Chemicals

Dowex AG 50W-X8 (200-400 mesh) Dowex AG 1-X8 (200-400 mesh) were obtained from Bio-Rad, Richmond, California, USA; amino acids, ninhydrin, diacetylmonoxime were obtained from Sigma Chemical Company, St. Louis, Missouri, USA; L-[U-<sup>14</sup>C]-arginine (120 mCi/mmol) from Bhabha Atomic Research Centre, Bombay. L-[U-<sup>14</sup>C]-ornithine was prepared from L-[U<sup>14</sup>C]-arginine as described previously (Gopalakrishna and Nagarajan, 1978).

Fibrosarcoma, obtained (approximate diameter 1.5 cm) after 15 days of transplantation was used.

For the estimation of enzymes, fibrosarcoma tissue was homogenized in 10 vol of 0.01 M Tris-HCl/0.15 M KCl/0.01 M MnCl<sub>2</sub> (pH 7.5). For the estimation of arginase and ornithine carbamoyl transferase, the whole homogenate was used. Ornithine transaminase was estimated after centrifugation of the homogenate at 600 g for 10 min. Ornithine decarboxylase was estimated after centrifugation of the homogenate at 13,000 g for 15 min.

### Arginase

Enzyme activity was measured as described previously (Gopalakrishna and Nagarajan, 1979b).

### Ornithine carbamoyl transferase

The assay mixture containing 0.2 ml of homogenate, 80 µmol triethanolamine-HCl (pH 7.4), 4 µmol ornithine, 20 µmol dilithium carbamoyl phosphate in a total volume of 1.0 ml was incubated at 37°C for 15 min. The citrulline formed was estimated (Gopalakrishna and Nagarajan, 1980c).

### Ornithine decarboxylase

This enzyme was assayed as described by Freidman *et al.* (1972). The assay mixture containing 50 mM Tris-HCl buffer (pH 7.8), 0.5 mM pyridoxal-5'-phosphate, 10 mM 2-mercaptoethanol, 2 mM-ornithine containing 600,000 cpm and 0.5 ml of enzyme preparation in a total volume of 1 ml was incubated at 37°C for an hour in a 10-ml Warburg's flask. The <sup>14</sup>CO<sub>2</sub> formed was trapped in 0.1 ml Soluene-350 (Packard Instrument Company, Downers Grove, Illinois, USA) and the radioactivity was measured.

### Ornithine transaminase

Prior to estimation of the enzyme activity, the homogenate was subjected to a centrifuge column technique to remove low-molecular-weight compounds (Gopalakrishna and Nagarajan, 1979a), and assayed according to the method of Herzfeld and Knox (1968). The homogenate (0.2 ml) was incubated with 50 mM phosphate buffer (pH

7.5), 35 mM L-ornithine, 5 mM  $\alpha$ -ketoglutaric acid and 0.05 mM pyridoxal-5'-phosphate in a total volume of 1 ml at 37°C for 20 min.

Unlike the previous workers, we estimated glutamic acid formed instead of glutamic- $\gamma$ -semialdehyde. After arresting the enzymic reaction by heating the reaction mixture in a boiling-water bath for 5 min, 0.1 ml of 0.2 M HCl was added and centrifuged at 1000 g for 5 min to remove denatured protein. The supernatant (0.5 ml) was applied to Dowex AG1 (acetate form, 1 ml bed volume) packed in 2 ml plastic syringe and ornithine and glutamic- $\gamma$ -semialdehyde were washed with 4 ml of water. Then glutamic acid was eluted with 2 ml 2 M acetic acid. To this fraction, 0.2 ml of 4 M NaOH was added and glutamate was estimated by the ninhydrin reaction (Moore and Stein, 1954).

All the enzyme activities were expressed as  $\mu$ mol of product formed/h/g tissue.

#### *Separation and estimation of metabolites*

Arginine and other related metabolites ornithine, citrulline, urea, proline, glutamate, glutamine and polyamines were separated from the tissue extract and estimated as described previously (Gopalakrishna and Nagarajan, 1980b).

Fibrosarcoma tissue (1 g) was homogenized in 10 ml of 5% perchloric acid and the precipitated protein was removed by centrifugation at 1000 g for 10 min. Plasma (1 ml) sample was deproteinized with 8 ml of 5% perchloric acid. The supernatant, about 7 ml, was adjusted to pH 6.0-6.5 with 5 M KOH and precipitated potassium perchlorate was removed in the cold by centrifugation. The supernatant was used for ion-exchange chromatography.

#### *Ion-exchange chromatography*

A 2 ml plastic syringe containing Dowex AG50W ( $\text{Na}^+$  form, 1 ml bed volume) was kept on the top of a column of Dowex AG 1 (acetate form, 1×10 cm) and another 2 ml plastic syringe with Dowex AG50W ( $\text{H}^+$  form; 1 ml bed volume) was attached to the bottom so that the sample applied would pass through all the three in the order mentioned.

The perchloric acid extract (5 ml) was neutralized and applied to a Dowex AG50W ( $\text{Na}^+$ ) resin and allowed to drain through all the three resins and washed with 5 ml of water. The columns were then disassembled and metabolites bound to each resin were eluted as described below.

The basic metabolites bound to Dowex AG50W ( $\text{Na}^+$ ) were eluted as follows: ornithine with 5 ml of 0.15 M sodium citrate-HCl (pH 6); arginine with 4 ml of 0.5 M sodium citrate-HCl (pH 6.5) and then polyamines with 4 ml of 0.3 M NaOH. The acidic metabolites were bound to the Dowex AG1 column. Glutamate was eluted with 28 ml of 0.05 M acetic acid and it appeared in the last 14 ml of eluate.

The neutral metabolites were bound to Dowex AG50W ( $\text{H}^+$ ). Urea was eluted with 12 ml water. Glutamine and proline were eluted with 4 ml of 2 M HCl; the first 1 ml of eluate was rejected and the next 3 ml fraction contained proline and glutamine. Citrulline was then eluted with 2 ml of 6 M HCl. All the separated fractions except glutamate contained other amines. It was, therefore, necessary to estimate these metabolites with special colorimetric or enzymatic methods.

### Ornithine

Out of five 1 ml fractions of eluate containing ornithine, the third and fourth fractions were found to contain about 90% of the applied ornithine and were used for ornithine estimation. This fraction contained lysine also and was estimated by the modified Chinard's method as described by us elsewhere (Gopalakrishna and Nagarajan, 1980a).

### Arginine

To 1.75 ml of fraction containing arginine, 0.1 ml of 0.6 M NaOH, 0.1 ml of 0.5 M sodium carbonate-bicarbonate buffer (pH 10.6) and 0.05 ml of arginase preparation (3 units) were added and incubated at 37°C for 10 min and ornithine formed was estimated as described previously (Gopalakrishna and Nagarajan, 1979c).

### Polyamines

The polyamine fraction obtained was adjusted to pH 2 to 3 with 4 M HCl, applied to Dowex AG50W ( $H^+$  form; 1 ml bed volume) and washed with 4 ml of 1 M HCl. Then polyamines were eluted with 4 ml of 6 M HCl and evaporated at 60°C. This fraction was subjected to high voltage electrophoresis (30 volts/cm for 60 min) using 0.1 M citrate-NaOH buffer (pH 4.3). Standards of polyamines were also treated in a similar way and stained with ninhydrin to locate the polyamines. Corresponding portions in other strips were cut and soaked in 5 ml of 0.3 M borate-NaOH buffer (pH 10 for putrescine, 10.5 for spermidine and spermine). After leaving at room temperature for 10 min, the samples were shaken for a minute for complete extraction of polyamines. From these samples, polyamines were estimated using fluorescamine (Veanling *et al.*, 1974).

### Glutamic acid

Glutamic acid was estimated using ninhydrin (Moore and Stein, 1954).

### Urea

Urea was estimated by the modified Archibald's method using diacetylmonoxime (Marshall and Cohen, 1972).

### Proline

To 1 ml of proline sample in 2 M HCl, 1.0 ml of 2 M NaOH, 2 ml of glacial acetic acid and 2 ml of Chinard's ninhydrin reagent were added and the colour development and measurement were done as described for arginine.

### Glutamine

The glutamine sample in 2 M HCl was converted to glutamate at 110°C for 2 h in a sealed tube and then evaporated at 60°C. The residue was dissolved in 2 ml of water and pH was adjusted to 6. Glutamate was estimated after ion-exchange chromatography on Dowex AG1 (acetate form, 1×10 cm) as described above.

### Citrulline

To 1 ml of citrulline in HCl, 0.5 ml of 10 M NaOH was added and citrulline was estimated using diacetylmonoxime and antipyrine (Ceriotti and Gazziniga, 1967).

### *Radioactivity measurement*

For radioactivity measurement, metabolites in the tissue homogenate were separated using ion-exchange chromatography as described above. Without further treatment, radioactivity present in ornithine and arginine fractions were measured. Glutamate and polyamine fractions were subjected to high voltage electrophoresis as described earlier and the identified portions of the paper strips were used for counting radioactivity. Proline and citrulline fractions were subjected to paper chromatography using a solvent system of phenol and water (5:2 w/v).  $^{14}\text{CO}_2$  from urea was measured by the urease reaction in a 10 ml Warburg's flask. To 1 ml of sample containing urea, 0.2 ml of 0.2 M Tris-HCl buffer (pH 7.2) and 0.2 ml of urease preparation containing sufficient activity to hydrolyze completely one  $\mu\text{mol}$  urea, were added and incubated at 37°C for 1 h and  $^{14}\text{CO}_2$  formed was trapped in Soluene-350 and radioactivity was measured.

In all samples  $^{14}\text{C}$  was measured in a scintillation 'cocktail' consisting of 120 g of naphthalene, 12 g of 2,5-diphenyloxazole (PPO) and 0.3 g of p-bis 2-(5-phenyloxazolyl)-benzene (POPOP) in 1000 ml 1,4-dioxan using a scintillation counter (PRIAS model PLD of Packard) with 90% efficiency for  $^{14}\text{C}$ . The quenching was corrected by the internal standardization method.

### *Measurement of arginine catabolism*

Mince of fibrosarcoma tissue (1 g) were incubated with 10 ml of Krebs improved Ringer II solution (Krebs, 1969) along with 5  $\mu\text{Ci}$  of L-[U- $^{14}\text{C}$ ]-arginine at 37°C for 90 min. The minces were separated by centrifugation and washed with 10 ml of ice-cold incubation medium and processed for the separation and estimation of the products of arginine metabolism.

### **Results and discussion**

Fibrosarcoma contained arginase activity comparable to the high levels present in the adult normal kidney (as shown in table 1). Ornithine carbamoyl transferase

**Table 1.** Activities of a few enzymes of arginine metabolism in fibrosarcoma.

Enzyme	Activity $\mu\text{mol}$ product/h/g tissue
Arginase	1920 $\pm$ 7.3
Ornithine transaminase	9.5 $\pm$ 0.9
Ornithine decarboxylase	0.183
Ornithine carbamyl transferase	N.D.

The values are the mean  $\pm$ S.D. of four different estimations except for ornithine decarboxylase where average of three estimations is given.

N.D. = Not detectable.

activity could not be detected using the method of Gopalakrishna and Nagarajan, 1980b). Ornithine decarboxylase activity was 3.5 times higher compared to the level in the normal adult liver (Friedman *et al.*, 1972). Ornithine transaminase activity was low in fibrosarcoma compared to the values reported for kidney and liver and equal to other tissues like spleen and brain (Herzfeld and Knox, 1968).

The concentration of metabolites in tissues depends on the rate of its uptake from plasma, synthesis and utilization. No significant change in the concentration of metabolites was observed in the plasma 15 days after transplantation of tumour in rats. The concentration of metabolites in fibrosarcoma and plasma are shown in table 2. The arginine content in fibrosarcoma was higher than in the plasma and

**Table 2.** Concentration of metabolites involved in arginine metabolism.

Metabolite	Fibrosarcoma	Plasma of tumour bearing rat	Tissue/plasma ratio
Arginine	0.175 ±0.017	0.124±0.01	1.4
Ornithine	0.0175±0.002	0.035±0.005	0.5
Urea	3.27 ±0.38	4.08 ±0.56	0.9
Glutamate	5.30 ±1.5	0.15 ±0.02	35.3
Glutamine	2.48 ±0.4	0.40 ±0.03	6.2
Proline	0.798 ±0.033	0.075±0.010	16.4
Putrescine	0.147 ±0.033	N.D.	—
Spermidine	0.876 ±0.025	N.D.	—
Spermine	0.596 ±0.013	N.D.	—
Citrulline	0.066 ±0.007	0.052±0.006	1.2

The values are expressed as  $\mu\text{mol/g}$  tissue or ml plasma and represent mean ±S.D. of four different estimations.

comparable to values reported for the normal spleen (Gopalakrishna and Nagarajan, 1979a). Ornithine concentration in fibrosarcoma was lesser than in kidney and liver and also plasma (Gopalakrishna and Nagarajan, 1980a). The occurrence of high levels of ornithine in plasma and low levels in fibrosarcoma suggests that it may be rapidly utilized for polyamine synthesis in this tissue. The molar ratio of spermidine to spermine is about 1.4. The polyamine levels in fibrosarcoma are low compared to the reported values for other growing systems like regenerating liver and hepatomas (Russel and Lombardini, 1971; Williams-Ashman *et al.*, 1972).

The content of urea and citrulline in fibrosarcoma were equal to the plasma levels. In the absence of ornithine carbamoyl transferase, the citrulline in fibrosarcoma may have been taken up from the plasma rather than being synthesized *de novo*. The proline content in fibrosarcoma was higher than in normal tissues.

Most of the arginase activity in fibrosarcoma is present in mitochondria (Gopalakrishna and Nagarajan, 1980d). But the relative distribution of arginine in mitochondria and cytosol is not known. It would be interesting to know the extent of conversion of arginine to ornithine in intact tissue and also whether ornithine is utilized in the mitochondria for proline and/or glutamate synthesis or in cytosol for polyamine synthesis. When minces of fibrosarcoma were incubated with L-[U-<sup>14</sup>C]-arginine, the radioactivity appeared in proline, glutamate, glutamine and polyamines but not

in citrulline (table 3). This data suggests that arginase may be involved in the utilization of arginine for proline, glutamate and polyamine syntheses in the tumour, although the possibility of proline and glutamate serving as precursors for ornithine and subsequently to polyamines cannot be ruled out.

**Table 3.** Distribution of radioactivity in metabolites derived from L-[U-<sup>14</sup>C]arginine.

Metabolite	cpm/g tissue	cpm/ $\mu\text{mol} \times 10^{-3}$
Ornithine	67,080	3050.0
Urea	36,600	11.0
Glutamate	40,000	8.0
Glutamine	46,000	7.4
Proline	71,870	141.0
Putrescine	3,080	25.6
Spermidine	6,050	7.4
Spermine	22,725	43.0
Citrulline	N.D.	—
Arginine	148,600*	1143.0

Minces of fibrosarcoma tissue weighing 1g were incubated with 10 ml of Krebs Improved Ringer II solution along with 5  $\mu\text{Ci}$  of L-[U-<sup>14</sup>C]arginine (specific activity 120 mCi/mmol) for 90 min at 37°C. Then centrifuged and the minces were processed. \* Not corrected for surface adsorption of arginine.

Tumour cell population is known to be heterogeneous. Some cells stay in the resting G<sub>0</sub> phase and some enter into proliferation. Volpe *et al.* (1974) observed a 'kidney' form of ornithine transaminase in the G<sub>1</sub> phase and a 'liver' form of ornithine transaminase in the S phase in HeLa cells and suggested that switching takes place between pathways involving ornithine conversion to proline and glutamate and vice versa. However, further studies are needed to know whether the cell population in fibrosarcoma has the potential to use proline and glutamate as precursors for ornithine.

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## A specific effect of copper on methylene blue sensitized photodegradation of nucleic acid derivatives

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MS received 9 May 1980; revised 3 September 1980

**Abstract.** Among several metal ions tested,  $\text{Cu}^{2+}$  was unique in slowing down methylene blue sensitized photodynamic breakdown of some nucleic acid bases and nucleosides. The  $t_{1/2}$  values were increased in the case of xanthine and uric acid by  $\text{Cu}^{2+}$ , but without any alteration in the nature or amounts of photoproducts formed. Xanthine was degraded quantitatively to allantoin and urea.

The breakdown of the sugar moiety of nucleosides was more drastically retarded than that of the purine ring. The decomposition rate and its magnitude was dependent on the concentration of  $\text{Cu}^{2+}$  as well as the nucleoside. The most profound increase in  $t_{1/2}$  values was found with xanthosine—7 min for the purine ring and 65 min for the ribose moiety, at 0.6 mM  $\text{Cu}^{2+}$ .

$\text{Hg}^{2+}$ , in the case of xanthine, and some paramagnetic metal ions in the case of the nucleosides, slowed down the photobreakdown to a small extent; however, differential effects were not observed unlike with  $\text{Cu}^{2+}$ . None of the other metal ions tested significantly influenced the process.

**Keywords.** Photodynamic inactivation; metal effects; methylene blue; purine photobreakdown.

### Introduction

Photodynamic inactivation of nucleic acids is brought about in the presence of oxygen by photosensitizing dyes. In polynucleotides, the base affected is guanine (Sivarama Sastry, 1968). A variety of purine derivatives structurally related to guanine, and several dyes have been studied in model systems with a view to understand the details of this process (Simon and Van Bunakis, 1962; Sussenbach and Berends, 1965; Wacker *et al.*, 1963). It is now evident that this is a very complex phenomenon involving the breakdown of the purine ring, the nature of the photo-products formed depending upon the purine derivatives as well as the dye involved. The nature of the lesions also depends upon the conditions employed. When these are comparatively mild, with moderate dye concentrations and light intensity, no strand cleavage occurs, but coding properties of poly (UG) are destroyed (Simon *et al.*, 1965) and likewise infectivity of TMV-RNA is lost (Sivarama Sastry and Gordon, 1966). Under drastic conditions, extensive depolymerization of polynucleotides is brought about photodynamically (Simon and Van Vunakis, 1962; Sivarama Sastry and Gordon, 1964; Freifelder *et al.*, 1961). The mechanisms involved were elucidated by the work of Waskell *et al.*, (1966) who showed that the likely lesion under mild photodynamic conditions is primarily depurination at guanine sites

Abbreviations used: UV, ultraviolet.

following the cleavage of the N-C glycosidic bond and that drastic conditions bring about destruction of ribose attached to guanine which is the probable lesion that results in strand scission.

The influence of metal ions on photodynamic inactivation has not been well studied. Sussenbach and Berends (1963) have shown that paramagnetic metal ions inhibit photodegradation of deoxyguanylic acid sensitized by lumichrome. Sastry and Gordon (1966) have shown that the inactivation of TMV-RNA sensitized by acridine orange is retarded by paramagnetic metal ions. Whether it is a generalized effect on photobreakdown is not known.

In the present study the effect of some metal ions on methylene blue sensitized photodegradation of several purine derivatives has been examined and it will be shown that  $\text{Cu}^{2+}$  exerts a selectively greater influence in inhibiting ribose and deoxyribose breakdown in nucleosides, in comparison with purine ring degradation.

## Materials and Methods

### Materials

Xanthine (E. Merck, Bombay), uric acid (Reanal, Budapest, Hungary), guanosine and xanthosine (US Biochemical Corporation, Cleveland, Ohio, USA) and 2'-deoxyguanosine (Fluka AG, Buchs, Switzerland) were used. Zinc-free methylene blue was product of Matheson, Coleman and Bell, New Jersey, USA. Metal salts used were  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ,  $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{NiSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{HgCl}_2$  and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  were of analytical grade (British Drug House, India).

### Methods

*Irradiation procedures and analytical technique:* Irradiation of purine compounds was performed with 2 mg of each compound in 4 ml volume in 50 mM  $\text{Na}_2\text{CO}_3$ - $\text{NaHCO}_3$  buffer (pH 9.45) in the presence of 0.03 mM methylene blue and 40,000 lux light intensity in a water jacketted test tube (15 mm internal diameter) with constant circulation of water at  $30 \pm 1^\circ\text{C}$  and aeration by bubbling air pre-saturated with buffer used, at a predetermined minimal optimal rate. Metal salts were added as required. Under the conditions employed, precipitation did not occur in any of the irradiated mixtures, upto the maximal concentrations of metal ions employed.

The destruction of the purine derivatives was routinely followed by measuring the ultraviolet (UV) absorbance at the following wavelengths; xanthine and xanthosine, 245 nm; 2'-deoxyguanosine, 250 nm; guanosine, 260 nm; and uric acid, 290 nm. Aliquots (0.1 ml) from irradiated mixtures were removed at intervals of time, diluted to 4 ml and UV absorbance measured.

Ribose remaining at various time periods was determined by the orcinol method in 0.1 ml aliquots as in earlier studies (Waskell *et al.*, 1966). Deoxyribose was determined in 0.2 ml aliquots with diphenylamine (Schneider, 1957).

Allantoin in irradiated solutions was estimated by the differential method of Nirmala and Sastry (1972). Uric acid interference was corrected by separately determining the uric acid content of the irradiated mixtures by measuring the absorbance at 290 nm.

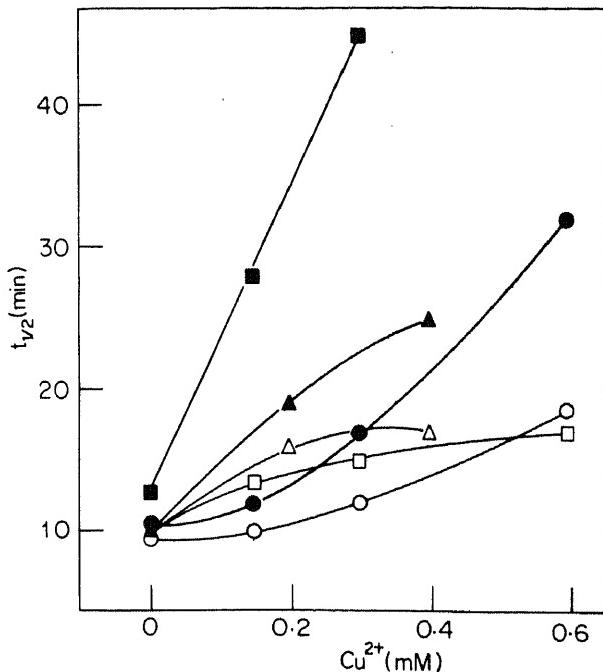
Urea was estimated with  $\alpha$ -isonitrosopropiophenone (Archibald, 1945).

Residual ultraviolet absorbance was plotted as a function of time and the time for

50% decrease in absorbance ( $t_{1/2}$ ) was determined graphically. When  $t_{1/2}$  could not be directly measured, it was extrapolated from the time course curves. In the case of nucleosides, ribose/deoxyribose destruction was also plotted graphically and the  $t_{1/2}$  for the sugar moiety determined separately.

## Results

Initial experiments showed that  $\text{Cu}^{2+}$  had a selective effect on retarding ribose breakdown in guanosine in comparison with the degradation of the ring. In view of this finding, the influence of varying concentrations of  $\text{Cu}^{2+}$ , as well as of other metal ions on photodynamic degradation of guanosine, 2'-deoxyguanosine and xanthosine was studied. The most dramatic effects were obtained with  $\text{Cu}^{2+}$  (figure 1).



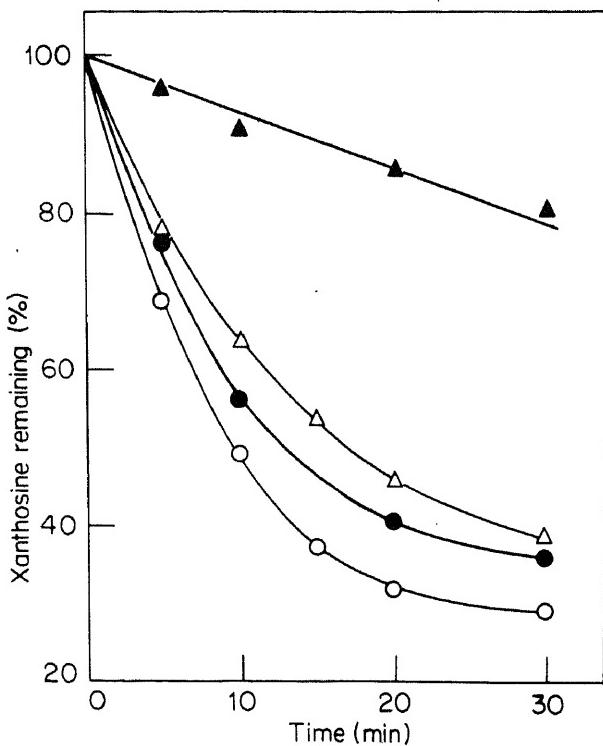
**Figure 1.** Effect of  $\text{Cu}^{2+}$  on photodynamic breakdown of guanosine, 2'-deoxyguanosine and xanthosine

Breakdown of the purine ring of guanosine ( $\Delta$ ), 2'-deoxyguanosine ( $\circ$ ) and xanthosine ( $\square$ ).

Breakdown of the sugar moiety of guanosine ( $\blacktriangle$ ), 2'-deoxyguanosine ( $\bullet$ ) and xanthosine ( $\blacksquare$ ).

Irradiation with 0.3 mM methylene blue in  $\text{Na}_2\text{CO}_3\text{-NaHCO}_3$  buffer (50 mM, pH 9.45) at 40,000 lux; Experimental details, see text.

The  $t_{1/2}$  values for guanosine ring breakdown were enhanced from 10 min to 17 min in the range 0-0.4 mM Cu $^{2+}$ ; in contrast, the increase in  $t_{1/2}$  for the ribose moiety of guanosine was from 10 min to 25 min. Higher concentrations of Cu $^{2+}$  could not be used, due to partial precipitation in the reaction mixtures. In the case of 2'-deoxyguanosine also, a similar concentration dependent effect of Cu $^{2+}$  was seen. With xanthosine the ribose destruction was very markedly inhibited, and  $t_{1/2}$  values for 0.6 mM Cu $^{2+}$  extrapolated from figure 2 gave a  $t_{1/2}$  for ribose of 78 min in contrast to a  $t_{1/2}$  for the purine ring of 17 min. Whereas  $t_{1/2}$  values are nearly identical for the ring and ribose moieties with guanosine and deoxyguanosine, with xanthosine normally ribose breakdown lags a little behind that of the purine ring ( $t_{1/2}$  values are 10 min for the ring and 13 min for ribose).



**Figure 2.** Effect of Cu $^{2+}$  on the time course of photodynamic breakdown of xanthosine.

Xanthosine irradiated in presence of 0.03 mM methylene blue with and without 0.6 mM Cu $^{2+}$ . Data indicate the disappearance of: (a) purine ring, control (O), with Cu $^{2+}$  ( $\Delta$ ); (b) ribose moiety, control (●), with Cu $^{2+}$  ( $\blacktriangle$ ) other details as in Fig. 1. Experimental details, see text.

Table 1 represents data obtained with various metal ions. Only  $\text{Cu}^{2+}$  is the most effective, and the other metal ions inhibit only very slightly and unlike  $\text{Cu}^{2+}$  do not exhibit differential effects on the disappearance of the ring and sugar moieties.

**Table 1.** Effect of metal ions on the photodynamic breakdown of guanosine 2'-deoxyguanosine and xanthosine

Compound (mM)	Metal ions	$t_{1/2}$ (min)	
		Purine ring	Ribose or deoxyribose
Guanosine (1.75)	None	10	10
	$\text{Cu}^{2+}$	17	25
	$\text{Co}^{2+}$	15	15
	$\text{Ni}^{2+}$	14	14
	$\text{Hg}^{2+}$	13	13
	$\text{Mg}^{2+}$	10	10
2'-Deoxy- guanosine (1.87)	None	9.5	11
	$\text{Cu}^{2+}$	18	32
	$\text{Co}^{2+}$	16	17
	$\text{Ni}^{2+}$	12	15
	$\text{Hg}^{2+}$	12	12
	$\text{Mg}^{2+}$	11	11
Xanthosine (1.75)	None	10	13
	$\text{Cu}^{2+}$	17	78
	$\text{Co}^{2+}$	14	22
	$\text{Ni}^{2+}$	13	17
	$\text{Hg}^{2+}$	15	17
	$\text{Mg}^{2+}$	11	14

The concentration of metal ions used were 0.4 and 0.6 mM for experiments with guanosine and 2'-deoxyguanosine/xanthosine respectively. Irradiation in the presence of 0.03 mM Methylene Blue in  $\text{Na}_2\text{CO}_3\text{-NaHCO}_3$  buffer (50 mM, pH 9.5) was carried out at 40,000 lux. Experimental details, see text.

To determine the effect of metal ions on photodegradable base derivatives, xanthine and uric acid were chosen. Similar experiments with several metal ions were carried out with xanthine and uric acid and the results obtained are shown in table 2. The concentrations of the metal ions used were the maximum possible levels that could be tested, since higher concentrations resulted in precipitation. Only  $\text{Cu}^{2+}$  was the most potent inhibitor but the nature of photoproducts was not altered by it. Of the other metal ions examined, only  $\text{Hg}^{2+}$  inhibited the photo-degradation of xanthine.

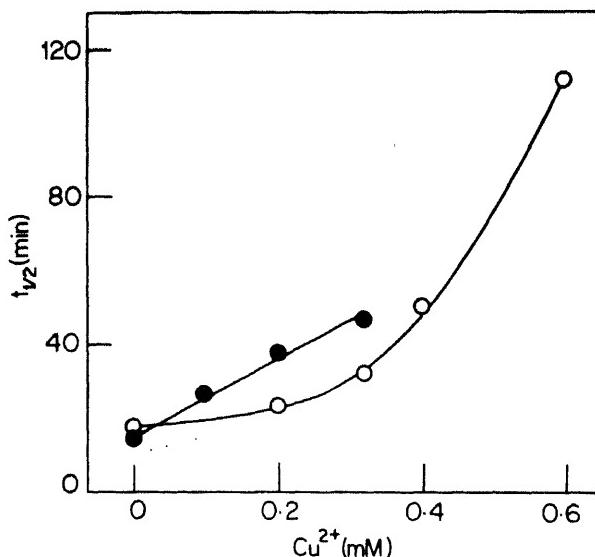
**Table 2.** Effect of metal ions on photodynamic degradation of xanthine and uric acid

Compound (mM)	Metal ions	$t_{1/2}$ (min)
Xanthine (3.29)	None	17.5
	Cu <sup>2+</sup>	112
	Co <sup>2+</sup>	18
	Ni <sup>2+</sup>	19
	Hg <sup>2+</sup>	26
	Mg <sup>2+</sup>	18
Uric acid (3.0)	None	15
	Cu <sup>2+</sup>	46
	Co <sup>2+</sup>	16
	Ni <sup>2+</sup>	13.5
	Hg <sup>2+</sup>	15
	Mg <sup>2+</sup>	15

Metal ions were included at 0.06 mM in the case of xanthine and at 0.03 mM for uric acid.

The samples in Na<sub>2</sub>CO<sub>3</sub>-NaHCO<sub>3</sub> buffer (50 mM pH 9.45) containing 0.03 mM methylene blue were irradiated at 40.000 lux.

The concentration dependence of the inhibitory effect of Cu<sup>2+</sup> on the photo-degradation of xanthine and uric acid is shown in figure 3. It is interesting that whereas the  $t_{1/2}$  of uric acid increased linearly with the concentration of Cu<sup>2+</sup> through the entire concentration range, with xanthine the effect was not very marked upto about 0.03 mM Cu<sup>2+</sup>, but thereafter it increased sharply.

**Figure 3.** Effect of Cu<sup>2+</sup> on photodynamic breakdown of xanthine and uric acid.

Uric acid, (●) and xanthine (○) irradiated with 0.03 mM methylene blue in presence of Cu<sup>2+</sup>  
Other details as in Fig. 1.

Since the photodynamic breakdown of uric acid sensitized by methylene blue has been shown to result in the formation of allantoin and urea as the two major photo-products (Nirmala and Sivarama Sastry, 1975), it was decided to examine the possible influence of  $\text{Cu}^{2+}$  on the formation of these two products. Irradiation for 30 min resulted in 95% breakdown of uric acid and 74% of xanthine (table 3).

**Table 3.** Effect of  $\text{Cu}^{2+}$  on the photodynamic breakdown of xanthine and uric acid.

Compound ( $\mu\text{mol/ml}$ )	$\text{Cu}^{2+}$ (mM)	Breakdown	Allantoin formed	Urea formed
			( $\mu\text{mol/ml}$ )	
Xanthine (3.29)	0	2.43	1.81	0.74
	0.03	1.17	0.88	0.26
Uric acid (3.00)	0	2.85	0.70	1.43
	0.03	0.77	0.21	0.48

Irradiation in the presence of methylene blue was done for 30 min with 0.03 mM in  $\text{Na}_2\text{CO}_3\text{-NaHCO}_3$  buffer (50 mM, pH 9.45) at 40,000 lux.

From these data, it is evident that the methylene blue sensitized photo-dynamic breakdown of xanthine results in the formation of allantoin and urea as the sole products, since they together fully account for the xanthine degraded.

## Discussion

The photodynamic breakdown of nucleic acids is retarded by paramagnetic metal ions (Sivarama Sastry and Gordon, 1964; Sussenbach and Berends, 1963). It had been suggested that this may be due to the metal ions shortening the life-time of the triplet state of the excited species (Sussenbach and Berends, 1963), but this does not completely explain the effects of metal ions observed in this study.  $\text{Cu}^{2+}$ , in particular, exerts a very unusual effect. Generally  $\text{Cu}^{2+}$  slows down purine ring breakdown, but with nucleosides,  $\text{Cu}^{2+}$  differentially and much more powerfully inhibits the photodegradation of the sugar moiety.

The results do not permit the postulation of a detailed mechanism by which  $\text{Cu}^{2+}$  exerts the very specific effect observed. As suggested earlier, a general effect on the triplet state is not likely to be involved since other paramagnetic metal ions do not have a similar effect. Neither is it likely that interaction of  $\text{Cu}^{2+}$  with hydroxyls at  $\text{C}_2'$  and  $\text{C}_3'$  is the determining factor since guanosine and 2'-deoxyguanosine are degraded similarly.

The  $\text{Cu}^{2+}$  effect on xanthine and uric acid photodegradation indicates that metal ion interaction with the purine ring is involved. In the case of guanosine, inosine and several related compounds,  $\text{Cu}^{2+}$  binding to N has been suggested, in addition to chelation with N and O<sub>6</sub> (Tu and Friedrich, 1968) though this is not fully accepted

(Eicchorn, 1973).  $Hg^{2+}$  has been thought to bind to N<sub>1</sub> of guanine at high pH and to the amino group at low pH (Simpson, 1964); N<sub>7</sub>-O<sub>6</sub> binding for  $Hg^{2+}$  has also been suggested (Yamane and Davidson, 1961; Eicchorn and Clark, 1963). Since Cu<sup>2+</sup> and Hg<sup>2+</sup> behave quite differently, from a quantitative point of view, the known complexing features imply that the site(s) of binding of metal ions to purine ring (and there could be more than one) under the present conditions also determine to a marked extent the influence of metal ions on photodynamic degradation of purine derivatives.

The degradation of ribose attached to a guanine site is important for an understanding of the mechanism of photodynamic depolymerization of polynucleotides. The present work suggests that metal ions such as Cu<sup>2+</sup> which protect against photodynamic inactivation, for example with TMV-RNA (Sivarama Sastry and Gordon, 1966), may do so by suppressing ribose breakdown and thereby chain cleavage; protection of guanine residues may be an additional factor.

Several metal ions (Ni<sup>2+</sup>, Co<sup>2+</sup> and Cu<sup>2+</sup>) are nearly equally effective in protecting TMV-RNA against photodynamic inactivation and these form ternary complexes containing RNA, dye and metal (Sivarama Sastry and Gordon, 1964, 1966). The present work shows that, of these, only Cu<sup>2+</sup> is a potent inhibitor of photodynamic breakdown of model compounds indicating that the specificity of interaction depends on the metal ion and the purine ring.

### Acknowledgement

We thank the University Grants Commission, Government of India for financial assistance.

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## **Preparation and properties of L-asparaginase from green chillies (*Capsicum annum L.*)**

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MS received 29 October 1979; revised 21 July 1980

**Abstract.** Green chillies (*Capsicum annum L.*) and tamarind (*Tamarindus indica*) contain appreciable amount of L-asparaginase. The enzyme was purified 400-fold from green chillies, by successive precipitations with ammonium sulphate and sodium sulphate, Sephadex-gel filtration and affinity chromatography and the purified enzyme was homogenous on gel electrophoresis. The enzyme exists in two forms, only one having antitumour activity.

The purified enzyme has a molecular weight of  $120,000 \pm 500$ . The N-terminal and the C-terminal amino acids are alanine and phenylalanine, respectively. The enzyme has a sharp optimum pH of 8.5 and a temperature optimum of  $37^\circ\text{C}$ . It is stable upto  $40^\circ\text{C}$ . The energy of activation is 3 kilo calories. The  $K_m$  value for the enzyme is 3.3. mM. The enzyme has little action on D-asparagine, which is a strong inhibitor. The enzyme has inseparable glutaminase activity and is thus an asparaginase—glutaminase. In addition, it possesses urease activity.

**Keywords.** Purification; *Capsicum annum*; L-asparaginase; asparaginase-glutaminase.

### **Introduction**

L-Asparaginase (L-asparagine amidohydrolase, E.C. 3.5.1.1) acquired some clinical importance in 1961, when the antitumour effect of guinea pig serum, originally discovered by Kidd (1953), was traced by Broome (1961) to the presence of this enzyme. The enzyme from *Escherichia coli* was clinically used to treat patients suffering from asparagine-dependent leukemias and lymphomas. A number of undesirable side effects, ascribed to the presence of contaminating bacterial endotoxins in the enzyme preparations were observed (Burchenal *et al.*, 1970). Hence, we attempted to prepare this enzyme from an entirely different source—plant materials—and study its biochemical and biological properties.

Very little work has been carried out on L-asparaginase from higher plants. Chibnall and Grover (1927) demonstrated the presence of an amidase in barley roots capable of hydrolyzing L-asparagine. Lees and Blackeney (1970) studied the distribution of L-asparaginase in *Lupinus leuteus* and *Dolichos lab lab* seedlings. A survey of several plants revealed that green chillies (*Capsicum annum L.*) contained appreciable amounts of this enzyme. In this paper, we report the partial purification and properties of the enzyme from green chillies.

## Materials and methods

The assay system (Meister, 1955) consisted of 0.5 ml of enzyme extract, 0.5 ml of substrate (L-asparagine, 0.04 M) and 1 ml of sodium borate buffer, 0.01 M, pH 8.5. After incubation for 1 h at 37°C, 1 ml of 15% trichloroacetic acid was added to stop the reaction. The ammonia formed was estimated by the method of Greenstein and Leuthardt (1958) by liberating it by the addition of a saturated solution of K<sub>2</sub>CO<sub>3</sub>, and aerating it into sulphuric acid traps (10 ml of 0.1 N H<sub>2</sub>SO<sub>4</sub>). To 5 ml of the resulting ammonium sulphate solution, 1 ml of Nessler's reagent was added. The intensity of colour was measured in a Klett-Summerson colorimeter using the 480 nm filter. Protein was determined by the method of Lowry (1951) using bovine serum albumin as the standard. One unit of L-asparaginase activity was defined as the amount of enzyme required to liberate 1 μmol of ammonia per mg protein per min.

### Purification of the enzyme from green chillies

Fresh green chillies (250 g) about 5-6 cm long and about 1-2 cm broad at the thick end, were homogenized with three volumes of 0.15 M KCl solution, centrifuged and the supernatant separated. This was designated as the crude extract. The residue was re-extracted with 0.15 M KCl solution and the total volume made up to 200 ml.

All the steps were carried out at 4°C.

To the crude extract, solid ammonium sulphate was added with stirring to give 60% saturation. The enzyme was collected by centrifugation at 7000 g, dissolved in 20 ml water and reprecipitated by the gradual addition of sodium sulphate to 30% concentration. The precipitate was dissolved in water and further purified by gel filtration through Sephadex G-75 using 0.15 M KCl as eluant (Sachs *et al.*, 1972).

The gel filtration yielded two isoenzymes corresponding to two peaks. As peak I enzyme was in larger amounts and also had a considerably higher specific activity (figure 1), it was taken for further purification by affinity chromatography.

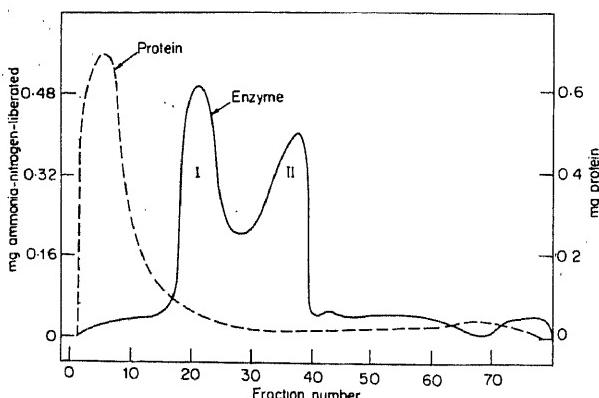


Figure 1. Sephadex G-75 chromatography of L-asparaginase.

CH-Sepharose-4B, covalently linked to D-asparagine was prepared according to the method of Kristiansen *et al.* (1970). The enzyme solution was applied to the affinity column (1×15 cm) and the unadsorbed proteins were washed away with the equilibrating buffer.

The above procedure resulted in a 400-fold purification with 28% recovery. The enzyme moved as a single band on polyacrylamide gel electrophoresis at pH 8.5.

The enzyme was dialyzed against distilled water and concentrated by slow evaporation in a vacuum desiccator. The enzyme powder thus obtained was stored at 4°C (table 1).

**Table 1.** Purification of L-asparaginase from green chillies (*Capsicum annuum L.*).

Purification step	Total units	Specific activity (units/mg. protein)	Fold purification	Recovery	Ratio of L-Asparaginase: L-glutaminase	Ratio of L-asparaginase: Urease
Crude extract	3200	0.4	—	100	2.3	2.56
Ammonium sulphate precipitation (60%)	2720	5.2	13	85	1.94	3.33
Sodium sulphate precipitation (30%)	1600	10	25	50	1.92	3.47
Sephadex G-75 filtration (I peak)	820	128	320	26	1.97	3.96
Sephadex G-75 filtration (II peak)	900	64	160	28	—	—
Affinity chromatography	480	160	400	15	2.05	3.93

### Properties

**Molecular weight determination:** Molecular weight of the enzyme was determined using Sephadex G-100 column calibrated with bovine serum albumin, cytochrome-C from horse heart, ribonuclease, aldolase and chymotrypsinogen.

**Determination of N-terminal and C-terminal amino acids in the purified L-asparaginase:** The N-terminal amino acid was determined using 1-fluoro-2, 4-dinitrobenzene (Sanger, 1945). The C-terminal amino acid was identified by hydrazinolysis (Akabori-Ohno, 1952).

### Results

The occurrence of L-asparaginase in various plant sources is presented in table 2. Chillies, tamarind, tomato etc. contain appreciable amounts of the enzyme; lemons, onions and potatoes have only trace amounts, while the enzyme could not be detected in drumsticks and ginger.

**Table 2.** L-Asparaginase in various vegetables.

Enzyme source	Activity ( $1 \times 10^3$ )
Green chillies ( <i>Capsicum annum L.</i> )	9.43
Red chillies ( <i>Capsicum annum L.</i> )	9.43
Tamarind ( <i>Tamarindus indica</i> )	4.57
Yam ( <i>Amorphophallus</i> )	4.57
Tomato ( <i>Lycopersicum esculentus</i> )	3.72
Radish ( <i>Raphanus sativus</i> )	3.43
Plantain ( <i>Musa paradisica</i> )	3.14
Brinjal ( <i>Solanum melongena</i> )	3.14
Cucurbita ( <i>Cucurbita maxima</i> )	2.86
Ladies fingers ( <i>Hibiscus esculentus</i> )	1.71
Mango ( <i>Mangifera indica</i> )	1.14

(Activity expressed as  $\mu\text{mol}$  of ammonia liberated by 1 g of fresh tissue in 1 min.).

#### *Physico-chemical and kinetic properties of the enzyme*

The molecular weight of the enzyme was determined to be approximately 120,000  $\pm$  500 daltons, with alanine as the N-terminal amino acid and phenylalanine as the C-terminal amino acid.

The enzyme functioned optimally at pH 8.5 and temperature 37°C. The enzyme activity was linear for 60 min. The  $K_m$  value for L-asparagine was 3.3 mM.

The enzyme hydrolyzed L-glutamine also, the activity being 50% of the activity with L-asparagine (table 3). On the other hand, D-asparagine was a potent inhibitor (table 3).

**Table 3.** Substrate specificity of L-asparaginase.

Substrate	Concentration (mM)	Enzyme activity (mg NH <sub>3</sub> liberated)	% Activity
L-Asparagine	40	0.27	100
L-Glutamine	40	0.13	50
D-Asparagine	40	0.02	7
D + L-Asparagines	40 each	0.06	23

The activity using L-asparagine was taken as 100.

The enzyme hydrolyzed urea also. In order to determine whether these activities are inherent properties of the enzyme or due to contaminating enzymes, the glutaminase and urease activities were determined at each step of the purification (table 1).

The ratio of L-asparaginase to L-glutaminase activities remained constant at a value of 2.0 (within limits of experimental errors). On the other hand, the ratio of L-asparaginase activity to urease activity increased gradually from 2.56 to 3.93, showing that the urease activity is at least partially separable.

At 20 mM, ions of heavy metals like silver, mercury and copper inhibited the enzyme activity very strongly (more than 90%). The enzyme was also inhibited by ferric chloride (90%) and to a lesser extent by zinc and cobalt ions (about 80%). On the other hand, the addition of magnesium chloride to the incubation mixture slightly enhanced the enzyme activity (20%). The enzyme was also inhibited by EDTA (20%), manganese and magnesium ions, magnesium being the most effective.

## Discussion

In higher plants, L-asparaginase has been found to be present in vegetables and we have achieved a 400-fold purification of the enzyme from green chillies. This enzyme was purified from many bacteria and from guinea pig serum through diverse procedures, (Wriston *et al.* 1973). We adopted affinity chromatography as the final step in the purification procedure, since it is highly specific and can be expected to give considerable enrichment of the enzyme. The homogeneity of the purified enzyme from green chillies is shown by a single band in polyacrylamide and agar gel electrophoreses.

In existing in two forms, the green chilli asparaginase resembles more the bacterial enzymes than mammalian. Asparaginases from *E. coli B* (Mashburn and Wriston, 1964); *E. coli K 12* (Schwartz *et al.*, 1966) and *Acinetobacter calcoaceticus*, all exist in two forms, but only one having antitumor potential. On the other hand, the guinea pig serum enzyme yields only one sharp peak. The exact differences between the different forms of the same enzyme have not been worked out.

**Table 4.** L-Asparaginase, L-glutaminase and urease activities of the green chilli enzyme at various stages of purification.

Purification step	L-Asparaginase activity	L-Glutaminase activity	Urease activity
Crude extract	0.046	0.020	0.018
Ammonium sulphate precipitation	0.120	0.062	0.036
Sodium sulphate precipitation	0.184	0.096	0.053
Sephadex G-75 filtration (I peak)	0.360	0.183	0.091
Affinity chromatography	0.413	0.201	0.105

(The substrates L-asparagine, L-glutamine and urea were used at a concentration of 40 mM. The enzyme activity is expressed as mg ammonia liberated per min.).

The properties of L-asparaginase from green chillies show a number of similarities and some sharp differences when compared with the properties of L-asparaginases from other sources (summarised in table 5). The molecular weight of the enzyme

**Table 5.** Properties of L-asparaginases from different sources.

Source	Mol. wt.	No. of sub units	Iso-electric point	pH optimum	K <sub>m</sub> value (mM)	Glutaminase activity	Blood clearance time (μ)	Anti tumour activity
Guinea pig serum	138,000 133,000	N.D.	3.6-4.5	7.5-8.5	0.072	Nil	26 h	+
<i>E. coli</i> B	130,000	4	4.9	8.6	0.013	slight	4.2 h	+
<i>E. carotovora</i>	135,000	4	8.8-8.9 7.6-8.6	N.A.	0.01	slight	4.1 h	+
<i>Citrobacter freundii</i> (3 enzymes present)	140,000	4	N.D.	7.5	0.029	Yes	7 h	+
<i>Acinetobacter glutamina sificans</i>	120,000 97,000	4	8.43	7.4	0.048	Yes (1.2 times)	0.9 h (13-18 h)	+
<i>Vibrio succinogenes</i>	146,000	4	8.74	7.3	0.048	Nil	N.D.	+
<i>Alkaligenes eutrophus</i>	150,000	4	8.6	N.A.	0.015	Yes	N.D.	+
<i>Serratia marcescens</i>	147,000 150,000 130,000	N.D.	6.0	6.8	0.1 0.012	N.D.	6.3 h (26-29 h)	+
<i>Fusarium tricinctum</i>	165,000	N.D.	5.18	8.0	0.52	Nil	Very rapid	-
<i>Pseudomonas</i>	122,000 118,000	N.D.	7.6	10.5	N.D. (1.3 times)	Yes	0.6-6.4 h	+
<i>Acinetobacter calcoaceticus</i>	105,000	4	5.2	8.6	2.0	slight	N.D.	-
Green chillies ( <i>Capsicum annuum</i> L.)	120,000	N.D.	N.D.	8.5	3.3	Yes	4 h	+

N.D. = Not determined; N.A. = Information not available

from green chillies was found to be  $120,000 \pm 500$ ; which closely resembled the enzymes from guinea pig serum or *E. coli*, most of which have a molecular weight around 130,000 daltons. L-asparaginase from green chillies had L-alanine at the N-terminal end and L-phenylalanine as the C-terminal amino acid and resembled the enzyme in *Erwinia aroides* (Staerk *et al.*, 1971) which had alanine as the N-terminal amino acid. The optimum pH of 8.5 for the green chilli enzyme resembled those of *E. coli* B and *A. calcoaceticus* which also had pH optima around 8.5 but differed from the guinea pig serum enzyme which showed a wide range of optimal pH from 7.5–8.5.

The K<sub>m</sub> value for the chilli enzyme is 3.3 mM. This was considerably larger than the K<sub>m</sub> values for most of the asparaginases from other sources, which are of the order of 0.01 mM. The enzyme from green chillies has thus a lesser affinity for the substrate L-asparagine. The enzyme from green chillies is actually an 'asparaginase–glutaminase' enzyme. A number of such enzymes with both glutaminase and asparaginase activities were purified from *Pseudomonas* (Greenberg *et al.*, 1964),

from *A. glutaminasificans* (Roberts *et al.*, 1972) and from *A. eutrophus* (Allison *et al.*, 1971). It is not certain whether the presence or absence of L-glutaminase activity in L-asparaginase is an advantage or hindrance in the treatment of cancer. It is believed that L-glutaminase by itself is an anti-tumour agent (Roberts *et al.*, 1970).

The presence of urease activity in the purified L-asparaginase preparation was totally unexpected. The urease activity was separable, at least partially. As such, the enzyme may not be a general amidase. We have examined a few commercial preparations of L-asparaginase (all from *E. coli*) and found urease activity. The presence of urease activity can have very adverse effects on patients. By hydrolysing blood urea, it can give rise to ammonia toxicity.

The experiments reported here show that magnesium is an activator for L-asparaginase from green chillies. The inhibition of the enzyme activity by heavy metal ions like  $\text{Ag}^+$ ,  $\text{Hg}^{2+}$ ; and  $\text{Cu}^{2+}$  is to be expected.

### Acknowledgements

We thank Dr S. Krishnamurthi for his keen interest and constant encouragement. The financial support from the Council of Scientific and Industrial Research, New Delhi for this project is acknowledged.

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## A comparative study of the composition of the collagens of the foot of two lamellibranch molluscs occurring in different habitats

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MS received 5 July 1980; revised 30 September 1980

**Abstract.** A comparative study of the collagen of the foot of *Lamellidens* Sp. and that of the unmodified part of the foot in *Mytilus edulis* shows marked differences in physical properties, amino acid composition and in the degree of stabilization. But both conform to type I collagen of vertebrates. In these respects, the latter shows agreement with the features characteristic of byssus collagen, which is highly crosslinked, involving dimers and trimers of tyrosine. It is suggested that such differences may reflect the different functions of the organs concerned, the foot of *Lamellidens* being a locomotory organ of the animal, while the foot of *Mytilus edulis* is modified for anchorage of the animal. The vestigial part though not morphologically modified shows the essential compositional characteristics of the byssus being a mere remnant of it.

**Keywords.** *Lamellidens*, *Mytilus edulis*, collagen, chain composition, dityrosine crosslinks, iodotyrosines.

### Introduction

In a recent study of the collagen of the byssus threads of *Mytilus edulis*, it has been reported that the peculiar features of the byssus collagen are attributable to its association with a non-collagenous protein forming a complex, stabilized by biphenyl links (Gowri *et al.*, 1977). This feature is a reflection of the functional needs of the organ which serves to anchor the animal to the substratum so as to prevent its displacement, due to wave action, in its natural habitat. In view of the above observation, it is of interest to study the collagen of the foot of an allied *Lamellidens* type which is unmodified, for the purpose of comparison with that of the vestigial part of the foot of *Mytilus*, which have not been characterized. Although a major part of the foot is modified to form the byssus threads, a small part retains its natural form. It is of interest to study whether it shows features similar to those reported in byssus. The results would throw light on the relation between structural modification and characteristics of collagen located in such structures. This is attempted in the present investigation.

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\* The work reported forms part of the thesis approved by the University of Madras for the award of the Ph.D. degree to the first author.

## Materials and methods

### Materials

The materials used in the present investigation Lamellidens (fresh water mussel) and *Mytilus edulis* were collected from ponds and lakes in Madras and the Madras harbour.

Pepsin (3. X crystalline), horse radish peroxidase, ammonium persulphate and sodium dodecyl sulphate used were obtained from Sigma Chemical Company, St. Louis, Missouri, USA. Whatman CM 32 microgranular cellulose was obtained from Whatman Biochemicals, Kent, U.K. All other chemicals were of the analytical reagent grade.

### *Isolation and purification of collagen*

The foot of fresh water mussel and *Mytilus edulis* were cut into small pieces and washed well with cold distilled water and stirred in 0.5 M sodium acetate overnight to remove adhering components. Then it was centrifuged and the residue was washed well with cold distilled water and extracted with 1 M NaCl, 0.05 M Tris-HCl, pH 7.4 followed by 0.5 M acetic acid. The residue was treated with pepsin (3 crystalline Calbiochem) in the proportion of 1:100 for 48 h. Further purification of these extracts was carried out according to the method of Piez *et al.* (1963).

### *Chemical analysis*

The collagen samples were hydrolyzed in 6 N HCl in sealed tubes at 110°C for 24 h. Amino acid composition was determined in amino acid analyzer, Beckman Spinco model 120°C (Spackman *et al.*, 1958). Hydroxyproline content of the test sample was determined by the method of Neuman and Logan (1950). Tyrosine was estimated according to the method of Ottaway (1958). Hexosamine was determined by the method of Elson and Morgan (1933). The total carbohydrate content of the sample was estimated by the phenol-sulphuric acid method (Dubois *et al.*, 1956). Tyrosine and its derivatives in the hydrolysate of the collagen samples were identified by paper chromatographic analysis as reported earlier (Archer and Gocker, 1952, Gmelin and Virtanen, 1959, Aeshbach *et al.*, 1976). The column chromatographic procedure for the detection of dityrosine was carried out as follows: standard dityrosine was prepared according to the method of Gross and Sizer (1959). An acidified sample was applied to a column of cellulose phosphate (1×50 cm) equilibrated with 0.2 M acetic acid. The elution was performed with 0.5 M sodium chloride in 0.2 M acetic acid. Five ml fractions were collected and monitored at 280 nm (Anderson, 1964). The presence of other tyrosine derivatives was ascertained using Biogel-P-2 column chromatography (Hunt, 1972).

### *Polyacrylamide gel electrophoresis*

The chain composition of the collagen sample was determined by the SDS-polyacrylamide gel electrophoretic procedure of Furthmayer and Timpl (1971).

### *Physical properties*

The shrinkage temperature of the collagen sample was measured, using a microshrinkage meter. The denaturation temperature ( $T_d$ ) was estimated by measuring viscosity at different temperatures between 20-40°C using an Ubbelhold viscometer.

For measurement of intrinsic viscosity, concentrations ranging from 0.025% to 0.15% were used and the value was obtained by extrapolation of the specific viscosities to C=0.

#### Separation of subunits

The procedure of Piez *et al.*, (1963) was used to separate the  $\alpha$  and  $\beta$  chains and their dimers in denatured collagen samples. 50 mg collagen in starting buffer (0.06 M sodium acetate, pH 4.8) was denatured by warming to 60°C for 15 minutes. The 1.8×15 cm column of CM-cellulose was maintained at 40°C and operated at a flow rate of 120 ml/h. Elution was carried out by means of a linear gradient containing 400 ml of starting buffer sodium acetate, pH 4.8 and an equal volume of the limiting buffer (the starting buffer contained 0.1 M NaCl). The fractions were monitored at 230 nm in an Unican SP 1800 ultraviolet spectrophotometer.

#### Results and discussion

The foot of *Mytilus edulis* yielded only 5% collagen when extracted with neutral salt as compared to about 10% of collagen extractable from the foot of fresh water mussel. With buffer of acid pH as much as 40% of collagen could be extracted from the foot of fresh water mussel, as against 25% of collagen from the foot of *Mytilus*. However, by treatment with pepsin about 45% of collagen could be extracted in the case of fresh water mussel and 35% could be solubilized in *Mytilus edulis* leaving behind 35% as a residue. The solubility properties indicate that collagen from fresh water mussel is relatively in a pure condition and not stabilized to the extent found in the foot of *Mytilus*. The values obtained for the shrinkage temperature and denaturation temperature of the two collagens were compared. The shrinkage temperature of the collagen of *Mytilus edulis* was higher (80°C) than that of fresh water mussel (70°). The denaturation temperature of *Mytilus edulis* was 35°C which was higher than that of fresh water mussel in which the value was 30°C. The intrinsic viscosity of the two collagens were found to be 14 dl/g and 12 dl/g respectively. Although the carbohydrate content of both comes within the range found in many invertebrate collagens, the value obtained for *Mytilus edulis* was lower (2%) than that of fresh (water mussel (4.5%).

Table 1 shows the amino acid composition of the two collagens. The total imino acid content of *Mytilus edulis* was 151 residues/1000 comprising of 75 residues of proline and 76 residues of hydroxyproline while the total imino acid content of fresh water mussel was 184 residues/1000. The glycine value of the collagen of *Mytilus edulis* was less than that of fresh water mussel suggesting its possible association with non-collagenous protein (Piez., 1967). This suggestion was supported by the comparatively high value for tyrosine (25 residues/1000) compared to that obtained with the collagen of fresh water mussel (5.8 residues/1000). But the hydroxylysine values were lower in *Mytilus edulis* than in the fresh water mussel. In the latter the hydroxylysine value amounted to 20 residues/1000 correlated with the increased value of carbohydrate (4.5%) suggestive of a higher degree of glycosylation. Another feature distinguishing the collagen of the foot of fresh water mussel and that of *Mytilus* was the difference in the values of histidine. It is suggested that the higher value in the latter is connected with more pronounced crosslinking. It is known that histidine plays a role in the crosslinking of collagen (Tanzer *et al.*, 1973).

**Table 1.** Amino acid composition of the collagens of the foots in fresh water mussel and *Mytilus edulis*.

Amino acids	Foot collagen of fresh water mussel	Foot collagen of <i>Mytilus edulis</i>
3-Hydroxyproline	..	..
4-Hydroxyproline	82	76
Aspartic acid	58	62
Threonine	25	36
Serine	39	37
Glutamic acid	101	91
Proline	102	75
Glycine	302	266
Alanine	80	124
Valine	22	30
Methionine	12	6
Isoleucine	30	25
Leucine	35	30
Tyrosine	6	25
Phenylalanine	8	14
Hydroxylysine	20	5
Lysine	17	38
Histidine	2	9
Arginine	58	50

Compositions expressed as residues/1000 amino acid residues.

In *Mytilus edulis*, the unmodified foot was rich in tyrosine. Gowri et al., (1977) showed the presence of dityrosine and halogenated tyrosine derivatives in byssus material, which are responsible for the ease of stabilization. As in the byssus material, the acid hydrolysate of the foot also showed the presence of a number of tyrosine derivatives. Some of them gave a bluish fluorescence when viewed in UV light. One of the spots was identified as dityrosine based on  $R_f$  values and by comparison with authentic sample of dityrosine. Dityrosine was absent in the foot of fresh water mussel, while it was present in the foot of *Mytilus edulis*. The presence of dityrosine was further confirmed by column chromatography on cellulose phosphate, as reported earlier (Gowri et al., 1977).

With a view to identify the nature of the other derivatives of tyrosine, the alkaline hydrolysates of the samples were subjected to specific colour tests. Both 3-iodotyrosine and 3,5-diodotyrosine were identified in the foot of *Mytilus edulis* from the paper chromatogram based on the  $R_f$  values as well as by comparison with authentic samples subjected to identical treatment as the test samples. The presence of these derivatives were further confirmed by column chromatography on Bio-Gel P-2 (Gowri et al., 1977).

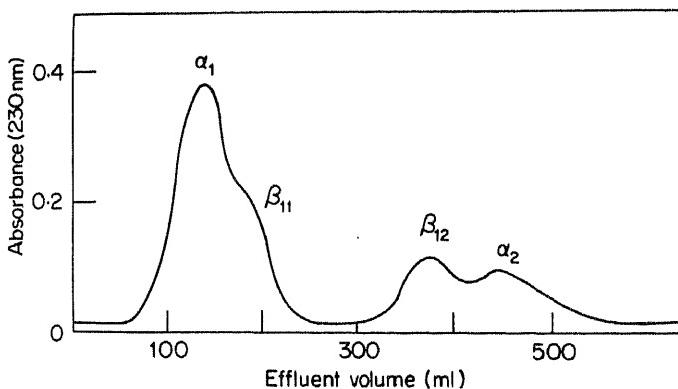
Although the test samples of the collagen of the foot of the fresh water mussel and that of the vestigial part of the foot of *Mytilus* are from organs which are homologous, they differ in the absence of tyrosine derivatives such as dityrosine and iodotyrosines, and in some physical properties as reported above. However, a study of the subunit composition of the two collagens by SDS-polyacrylamide gel electrophoresis showed

that both belonged to type I collagen of vertebrates (figure 1) similar to the sub-unit composition of the collagen of the rat tail tendon. The results of CM-cellulose chromatography of denatured samples of both the collagens support the above suggestion (figures 2a and 2b).

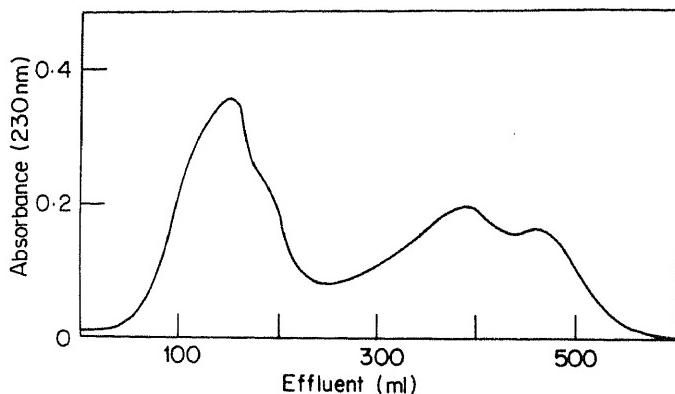
The differences noted in the two collagens may be in the nature of post-translational changes, to suit the performance of different functions. It is known that the functional role of collagens may be reflected in their chemical organization and physical properties, notwithstanding their molecular organization which is indicative of their genetic



**Figure 1.** SDS-polyacrylamide gel 5% electrophoretic pattern of acid-solubilized collagens of foot of *Mytilus a edulis* and fresh water mussel compared with that of rat tail tendon. (a) Rat tail tendon collagen (RTT); (b) Foot collagen of *Mytilus edulis*; (c) Foot collagen of fresh water mussel.



**Figure 2a.** CM-cellulose elution pattern of denatured acid soluble collagen of foot of fresh water mussel.



**Figure 2b.** CM-cellulose elution pattern of denatured acid-soluble collagen of foot of *Mytilus edulis*.

origin (Collins *et al.*, 1977). The significance of the difference between the collagens of the foot of fresh water mussel and that of the foot of *Mytilus*, is that they are correlated with the respective functions of the two organs in which they are located. The former is involved in locomotion by ploughing through the soft mud in the natural habitat, while the foot of *Mytilus* is a vestigial part left after the foot has undergone modification into byssus threads. The unmodified part of the foot shows the physico-chemical properties of the byssus. A degree of similarity in the amino acid composition and mode of stabilization of collagen of the foot and byssus threads, is attributable to the fact that both are modifications of the foot.

### Acknowledgements

The authors are grateful to Dr. N. Ramanathan, for his generous support and encouragement during this investigation.

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## **Effect of ethrel treatment on the postharvest changes in the turnover of ascorbic acid and reducing sugar in *Achras sapota* fruits**

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MS received 12 May 1980; revised 15 September 1980

**Abstract.** There was a definite relationship between growth and ascorbic acid content in *Achras sapota*. Increase in ethrel concentration from 250 ppm to 500 ppm hastened early ripening and increased the amount of reducing sugars but depleted the ascorbic acid content. Other aspects of ascorbic acid turnover viz. ascorbigen, bound form of ascorbic acid, ascorbic acid utilization, net ascorbic acid bound and ascorbic acid oxidase were also studied.

**Keywords.** *Achras sapota*; ascorbigen; ascorbic acid utilization; ascorbic acid oxidase; ethrel; fruit ripening; reducing sugar.

### **Introduction**

Ascorbic acid present in abundant amounts in fruits undergoes rapid oxidation to dehydroascorbic acid. The fruit, is also a rich source of carbohydrate, of which the most important one is sucrose, which is hydrolyzed to glucose; a precursor of ascorbic acid. Moldtmann (1939) has described ascorbic acid as a transformative product of the sugar first formed in photosynthesis.

Ethrel is a natural ripening agent of fruits (Kadev *et al.*, 1978). On dipping the mature fruits in ethrel, it enters fruit cells, releases ethylene and hastens the ripening process. Changes in ascorbic acid content may occur with ethrel spray which influences the carbohydrate metabolism in related fruits (Gopal and Vasudeva, 1975).

Contrasting reports are seen in the literature regarding the appearance and disappearance of ascorbic acid in different fruits during ripening (Hulme, 1974; Goldschmidt, 1977; Kadev *et al.*, 1978).

In the present study an attempt has been made to understand the ascorbic acid turnover in untreated fruits during the growth of fruits as well as in the fruits treated with ethrel (2-chloroethyl phosphonic acid) at concentrations of 250 ppm and 500 ppm with a view to study the ascorbic acid metabolism during the development and ripening of fruits. An attempt has also been made to study the content of reducing sugar in the fruits so as to know the relationship between the reducing sugar and ascorbic acid at the various stages of fruit development.

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\* since deceased

## Materials and methods

Chikku (*Achras sapota*) fruits were harvested from plants grown in the Horticultural section, Gujarat Agriculture University, Anand. Well matured fruits with similar shape and an average volume of 83.4 ml (volume in ml of water displaced) were selected for the experimental work. Fruits of different sizes varying from small, immature to big mature (at 5 different stages with varying volumes—10.3, 22.6, 31.1, 52 and 83.4 ml) were selected for the developmental studies. The fruits were washed and drained prior to ethrel treatment; a dip treatment of 1 min was given to all fruits. Control fruits were dipped in distilled water. Two different concentrations of ethrel, 250 ppm and 500 ppm, were used for the treatment. The control and ethrel treated fruits were separately placed in air tight plastic desiccators for further ripening. KOH pellets were placed in the desiccators to remove the CO<sub>2</sub> evolved during the respiration of the fruits. All the desiccators were placed at 24 + 1°C in a Gallenkamp incubator. Biochemical estimations were done at 24 h intervals for 5 days. Desiccators were aerated every day for about 1 h. The biochemical estimations for the experiment on the developmental studies were initiated from the day of harvest.

The reducing sugar content was estimated by the method of Lindsay (1973). Ascorbic acid, bound form of ascorbic acid, net ascorbic acid bound and ascorbic acid utilized were estimated using an Erma Photochem Colorimeter following the method of Chinoy *et al.* (1976).

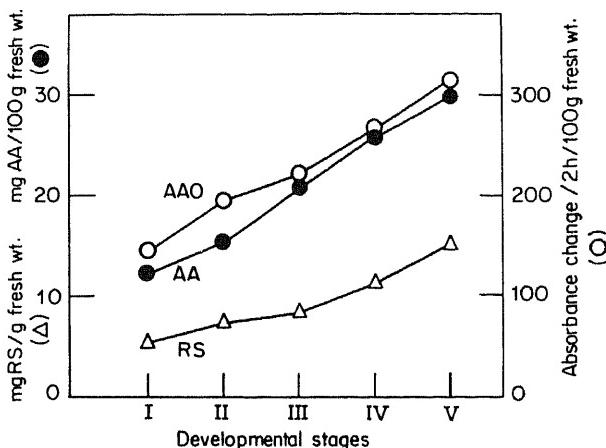
Ascorbic acid oxidase (EC 1.10.3.3.) was determined by measuring the ascorbic acid oxidized. The reaction mixture contained 1 mg ascorbic acid, 200 µmol acetate buffer (pH 5) and a suitable concentration of enzyme extract in a final volume of 7 ml. Incubation was carried out at 37°C for 2 h and the enzyme reaction was stopped by adding 2 ml of 5% metaphosphoric acid. Precipitated protein was filtered off and 2 ml of filtrate was used for the estimation of ascorbic acid (Chinoy *et al.*, 1976).

Concentration of ascorbic acid, bound form of ascorbic acid, net ascorbic acid bound were calculated as mg/100 g fresh wt., ascorbic acid utilized as %, ascorbic acid oxidase as absorbance change/100 g fresh wt/2h and reducing sugar as mg/g fresh wt. All the experiments were repeated thrice and a mean value was taken for calculation.

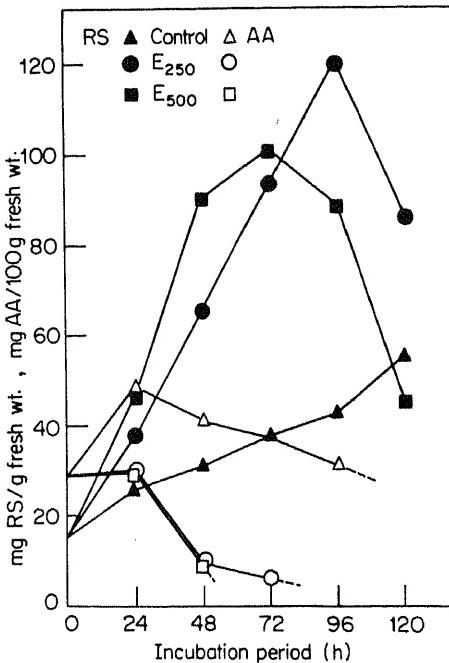
## Result

Ascorbic acid content increased during all the stages of growth till maturity (figure 1), decreased to very low values as the fruit entered the ripening phase and disappeared totally upon senescence of the fruit (figure 2). The decrease in ascorbic acid content was more rapid in the treated fruits than in the control and the decrease in the rate was enhanced with the increase in ethrel concentration (figure 2).

The content of reducing sugar increased as the stage of development advanced (figure 1). The quantity of reducing sugar reached a maximum value after 72 h in fruits treated with 500 ppm of ethrel and after 96 h in fruits treated with 250 ppm of ethrel and thereafter its level observed (figure 2). However, the highest content in reducing sugars was observed in the fruits treated with 250 ppm of ethrel.

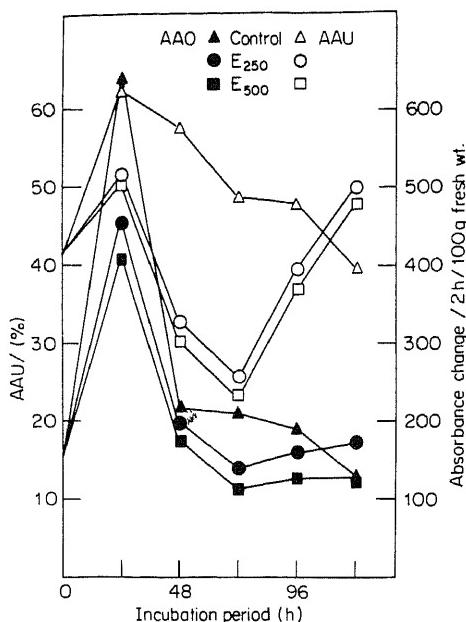


**Figure 1.** Level of ascorbic acid, (AA); reducing sugar, (RS); and ascorbic acid oxidase, (AAO) during the development of fruits.

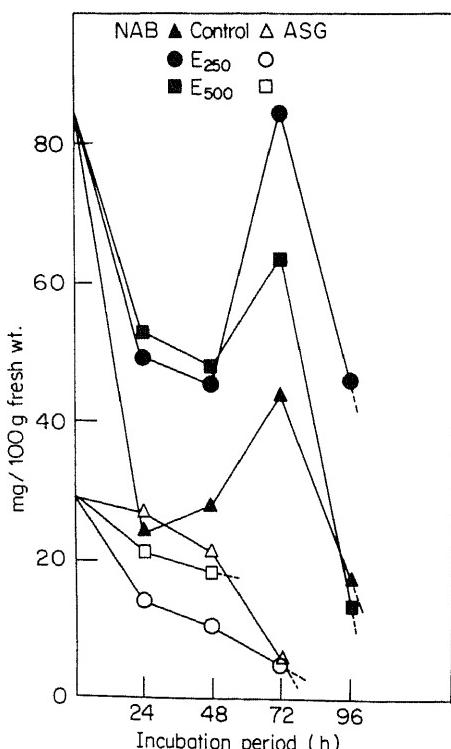


**Figure 2.** Levels of ascorbic acid and reducing sugar during the ripening of fruits.

The increasing activity of ascorbic acid oxidase and the high content of ascorbic acid were on par during the development of fruit (figure 1). The amount of ascorbic acid utilized and ascorbic acid oxidase level were depleted along with the decrease in ascorbic acid during the ripening, but the treated fruits showed fluctuations in the utilization of ascorbic acid and ascorbic acid oxidase (figure 3). The trend in the changes of ascorbic acid bound was the same as that of ascorbic acid, but net ascorbic acid bound showed fluctuation during ripening (figure 4).



**Figure 3.** Levels of ascorbic acid utilization, (AAU); and ascorbic acid oxidase, (AAO) during the ripening of fruits.



**Figure 4.** Levels of ascorbic acid bound, (ASG); and nett ascorbic acid bound, (NAB) during the ripening of fruits.

## Discussion

The results revealed a positive correlation between the size of the fruit and its ascorbic acid content. A similar relationship between ascorbic acid content and fruit size was established by many researchers (Sako, 1973; Nag *et al.*, 1974; Rousi and Aulin, 1977).

The rise in ascorbic acid content during the growth of the fruit was concomitant with the increase of reducing sugars (Barker and Mapson, 1952). Since ascorbic acid is biosynthesized from sugars (Loewus, 1959; Chinoy, 1967) the increase in ascorbic acid was related with the increase in reducing sugars which was linked with the enhanced photosynthetic activity of the young fruits (Gasporgan, 1950; Nagi, 1951).

A decrease in ascorbic acid content observed during and after ripening of chikku suggested that it was catabolized faster to meet the increased respiratory requirements during ripening. This result also suggested that the ripening shifted the redox balance to the oxidative side. An increase in reducing sugar during climacteric occurred by the hydrolysis of various polysaccharides. The decrease in ascorbic acid was reported in different fruits by other workers (Trofimov, 1967; Rousi and Aulin, 1977).

Post-harvest treatment with different concentrations of ethrel is found to influence the total carbohydrate metabolism in the ripening sapota fruit (our unpublished data). Since the quantitative changes of ascorbic acid depended mainly on carbohydrate metabolism (Isherwood and Mapson, 1962) the ethrel treatment was indirectly connected with the ascorbic acid metabolism. The accelerated ripening and enhanced rate of ascorbic acid degradation on increasing the ethrel concentration suggested that these two processes may be linked.

The incubation of fruits at  $24 + 1^{\circ}\text{C}$  might have accelerated the synthesis of sugars. Barker and Mapson (1952) reported that the transfer of tubers from 10 to  $25^{\circ}\text{C}$  increased the synthesis of sucrose initially and changed the ratio of sucrose/hexose sugars. A similar trend in ascorbic acid and oxidase levels during growth until climacteric suggested a continuous synthesis as well as utilization of ascorbic acid during this period. The growing fruits require more energy with greater turnover of ascorbic acid (Chinoy, 1969). The decline in ascorbic acid, the oxidase and the acid utilized during ripening and the upsurge in ascorbic acid oxidase and the amount of ascorbic acid utilized and the decrease in reducing sugar after climacteric may presumably due to the respiratory requirements. Among all the enzymes metabolizing ascorbic acid, ascorbic acid oxidase has a vital role in ripening process (Mandals, 1953; Ward, 1955). The activity of ascorbic acid oxidase was reported to be the highest at pH 5 in apple fruit (Ponding and Joslyn, 1948) which was also observed in sapota fruit. Moreover the sudden appearance of peroxidase (our unpublished data) at the climacteric rise may also be a causal factor for the quick oxidation and disappearance of ascorbic acid during ripening. The chikku fruits reached the climacteric stage after 5 days in the control and 4 days and 3 days in the cases treated with 250 ppm and 500 ppm of ethrel respectively, indicating a positive effect of ethrel towards the early ripening of fruits.

The disappearance of bound forms of ascorbic acid at a later stage of ripening might be because of their hydrolysis and release to meet the increased demand for ascorbic acid.

### Acknowledgements

We thank the University Grants Commission, New Delhi and Gujarat Government for financial assistance. Thanks are also due to Agromore Ltd., Bangalore for providing ethrel as a free gift.

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## **Effect of L-cystine on macromolecular changes during spore and parasporal crystal formation in *Bacillus thuringiensis* var. *thuringiensis*.**

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MS received 4 August 1980; revised 22 October 1980

**Abstract.** High concentration of L-cystine (0.25%) when present in a glucose-mineral salt medium inhibited sporulation-specific events like protease production, calcium uptake and dipicolinic acid synthesis in *Bacillus thuringiensis* var. *thuringiensis*. In addition, the enzymes of the Krebs cycle from aconitase onwards were completely inhibited by a high concentration of cystine. At a low concentration of cystine (0.05%), none of the above mentioned macromolecular changes were affected. Lipid synthesis monitored by [1,2<sup>14</sup>C]-acetate incorporation into lipid as well as into whole cells was completely inhibited.

**Keywords.** *Bacillus thuringiensis*; spore; crystal; macromolecular changes.

### **Introduction**

Bacterial sporulation is considered to be one of the best model systems to study cellular differentiation. The biochemical and genetic changes which take place during sporulation have been well studied in several bacilli (Baillie and Norris, 1964; Bernlohr and Novelli, 1963; Bulla *et al.*, 1971; Powell, 1953). Some of the events have been implicated as a prerequisite for sporulation, because of their temporal relationship to the morphological development of the spores, or because of their absence in certain mutants unable to form spores. Several such physiological phenomena were observed in aerobic spore formers (Baillie and Norris, 1964; Schaeffer, 1969). The overall pattern of metabolism was well studied in *B. thuringiensis* (Nickerson and Bulla, 1975; Nickerson *et al.*, 1974; Somerville, 1971) and it bears a similarity to that of *Bacillus cereus* (Hanson *et al.*, 1963).

*B. thuringiensis* is toxic to lepidopterous insect larvae (Bulla *et al.*, 1977) through the elaboration of a glycoprotein crystal within the cell (Bechtel and Bulla, 1976). The time of appearance of the spore and the crystal overlap (Bechtel and Bulla, 1976). Physical and chemical studies as well as electron-micrography provided a considerable amount of information on the structure and formation of spore and

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Abbreviations: cys/cysSH, cystine and cysteine; TLC, thin layer chromatography.

crystal protein (Bechtel and Bulla, 1976; Bulla *et al.*, 1977; Holmes and Monro, 1965; Somerville, 1971). Immunological studies also proved the identity of the major spore coat protein and the crystal protein of *B. thuringiensis* (Lecadet *et al.*, 1972; Somerville *et al.*, 1970). Loss of the ability to produce crystal protein led to the loss of spore coat (unpublished data) and consequent lysozyme sensitivity (Cheng and Aronson, 1977; Stahly *et al.*, 1978). It was proposed that both spore and crystal formation occurred after the cessation of vegetative growth (Yousten and Rogoff, 1969); and it was established that the crystal protein was synthesized after the stationary phase (Monro, 1961).

In several bacilli, the process of sporulation and germination was shown to be nutritionally-dependent (Hardwick and Foster, 1952). It was observed that L-cystine supported growth, sporulation and crystal formation in *Bacillus thuringiensis* var. *thuringiensis* (Nickerson and Bulla, 1975; Rajalakshmi and Shethna, 1977; and previous paper). These results suggest that cystine might interfere with some of the macromolecular changes during sporulation and parasporal crystal formation. This paper describes the effect of cystine on several macromolecular processes.

## Materials and methods

### Organism and culture conditions

*Bacillus thuringiensis* var. *thuringiensis* serotype I was obtained from Prof. H. de Barjac, Institute Pasteur, Paris, France. Minimal medium containing mineral salts and 1% glucose and the inoculum were prepared as described before (Rajalakshmi and Shethna, 1977). For further studies, the mineral medium was supplemented with low (0.05%) and high (0.25%) concentrations of cystine; cells grown with low concentration of cystine were considered as control, and those with high concentration of cystine as experimental.

Growth was monitored by following the absorption at 600 nm in a Bausch and Lomb Spectronic-20 colorimeter. Spore and crystal formation were monitored by phase contrast microscopy (Zeiss phase contrast microscope, Germany). Toxicity was checked by feeding the cell debris (12,000 g, 10 min) to second or third instar larvae of silkworm moth (*Bombyx mori*). After three to four h of feeding, the mortality was scored. Feeding was done by spraying the toxin (1 mg per leaf) on mulberry leaf and feeding the leaves to the silk worms (20 worms per leaf).

For studying calcium uptake, aspartokinase activity, dihydrodipicolinic acid synthase (EC 4.2.1.52) activity, and the enzymes of the Krebs' cycle, the cells were harvested at 30 min intervals from 1 h before they reached the stationary phase.

Cell-free extracts, wherever necessary, were prepared by subjecting the cells to sonication (30 sec×8) followed by centrifugation at 4°C (12,000 g for 10 min).

### [<sup>45</sup>Ca] uptake by cells

The uptake of Ca<sup>2+</sup> was monitored by using [<sup>45</sup>Ca<sup>2+</sup>]. Control and experimental cells were grown in 200 ml of medium in 500 ml Erlenmeyers flasks, incubated on a rotary shaker (250 rpm) at 30°C. Two h prior to the onset of the stationary phase, 10 ml samples were distributed into sterile tubes. Radioactive calcium [<sup>45</sup>Ca] in HCl (0.1mCi) was diluted to 50 ml with sterile 0.05M potassium phosphate buffer pH 7.2.

The diluted [ $^{45}\text{Ca}$ ] (0.5 ml) was added to the tubes containing 10 ml of the culture and incubated on a rotary shaker at 30°C. One h prior to the stationary phase, the tubes containing the cultures were removed in duplicate both from the control and from the experimental sets and processed separately as follows. The cells were washed thrice and suspended in 5 ml of 0.05 M potassium phosphate buffer pH 7.2. The cell suspension was broken by sonication; 0.1 ml of the sonicated material was spotted on a Whatman No. 3 filter paper, dried and counted in a liquid scintillation counter.

#### *Dipicolinic acid synthesis:*

Dipicolinic acid was extracted from the control and experimental cultures at 30 min intervals from 0 to 10 h of the stationary phase according to the method of Hoganson and Stahly (1975). Estimation of dipicolinic acid was carried out according to the method of Rotman and Field (1968).

#### *Enzymes of dipicolinic acid synthesis*

- a. *Aspartokinase (EC 2.7.4.4)* The activity of the enzyme aspartokinase was measured according to the standard procedure (Truffa-Bachi and Cohen, 1970).
- b. *Dihydrodipicolinic acid synthase (EC. 4.2.1.52)* The assay for dihydrodipicolinic acid synthase was carried out by a modification of the *O*-aminobenzaldehyde procedure (Stahly, 1969).

#### *Metalloprotease activity*

The activity of metalloprotease in the culture medium was determined by the digestion of azoalbumin according to the method of Li and Yousten (1975).

#### *Enzymes of Krebs' cycle*

The crude extracts of the cells were used for the various enzyme assays. Pyruvate dehydrogenase (EC. 1.2.2.2; EC. 1.2.4.1) activity was measured according to the method of Reed and Willms (1966). The assay mixture to measure the activity of citrate synthase (EC. 4.1.3.7) was prepared by the standard procedure (Weitzman, 1969). The aconitase (EC. 4.2.1.3) activity was measured spectrophotometrically at 240 nm by following the disappearance of *cis*-aconitate (Fensler and Lowenstein, 1969). The method to measure the activity of isocitrate dehydrogenase (EC. 1.1.1.41) was based on the measurement of the increase in the absorbance at 340 nm (Cleland *et al.*, 1969). The assay for  $\alpha$ -ketoglutarate dehydrogenase was carried out by the standard procedure (Mukherjee *et al.*, 1965). The reaction mixture for succinate dehydrogenase (EC. 1.3.99.1) was prepared according to Veeger *et al.*, (1969). The activity of fumarase (EC. 4.2.1.2) was calculated by measuring the changes in fumarate concentration by following absorbance at 250 nm (Hill and Bradshaw, 1969). The assay for malate dehydrogenase (EC. 1.1.1.37) was carried out by measuring the increase in adsorbance at 340 nm due to the reduction of NAD (Yoshida, 1969).

#### *Lipid synthesis*

Lipid synthesis was monitored by following [1, 2,  $^{14}\text{C}$ ]-acetate incorporation into the lipid moiety. Fifty ml cultures were labelled with 0.1 mCi of [1,2,  $^{14}\text{C}$ ]-acetate. The cells were harvested at 60 min intervals and the lipid extraction was carried out by

the standard procedure (Bulla *et al.*, 1970). Since acetate incorporation was negligible in the mid logarithmic phase, lipid synthesis at this stage was characterised as follows. Cells from 50 ml of the mid log culture were centrifuged (8000 g for 10 min) washed thrice with 0.05 M potassium phosphate buffer pH 7.2, and transferred to 50 ml of fresh mineral salt medium containing no glucose but 0.1 mCi of [1,2 <sup>14</sup>C]-acetate. After 30 min of incubation on a rotary shaker at 30°C, the cells were harvested and the lipids were extracted and characterized as before.

#### *Lipid extraction*

The method for the qualitative and quantitative analysis of neutral lipids was based on the ability of lipids to reduce acid-dichromate,  $\text{CrO}_7^{2-}$  to  $\text{Cr}^{3+}$  (Bragdon, 1951). Phospholipids were analysed and characterised according to the method of Barlette (1959).

#### *Autoradiography*

The lipid moieties labelled with [1,2 <sup>14</sup>C]-acetate were separated by thin layer chromatography (TLC), (Bulla *et al.*, 1970). One set of plates were developed by ninhydrin and iodine vapour. Another set of TLC plates were exposed to X-ray film (Indu X-ray films, Ootacamund) for 15 days and developed so as to visualize the radioactive spots. The radioactive spots were scraped off from the TLC plates and the lipid was extracted into chloroform-methonal (2:1 v/v); aliquots were counted in a Beckman LS-100 liquid scintillation spectrometer using a toluene-based scintillation fluid, to measure the radioactivity in each spot.

#### *Uptake of [1,2, <sup>14</sup>C]-acetate*

To 50 ml of log culture i.e., 9 h prior to the stationary phase, were added 0.25 mCi of [1,2, <sup>14</sup>C]-acetate. The cultures were incubated on a rotary shaker at 30°C. Five ml samples were removed at 1 h intervals and centrifuged (8000 g for 10 min), washed and suspended in 0.5 ml of 0.01 M potassium phosphate buffer, pH 7.2. The cell suspension was broken by sonication. 0.1 ml of the sonicated material was applied on a Whatmann No. 3 filter paper, dried and the radioactivity measured.

#### *Specific activities*

Uptake of [<sup>45</sup>Ca<sup>2+</sup>] was expressed as cpm per mg protein. The specific activities of all the enzymes studied were expressed as units per mg of protein. Metalloprotease activity was expressed as units per ml culture. Dipicolinic acid content was expressed as µg dipicolinic acid/10 ml culture. Total lipid content was calculated as percentage of the total dry weight of the cells. Phospho- and neutral-lipid contents were expressed as per cent of the total lipid content of the cell. Acetate uptake into the whole cells was expressed as cpm per mg dry weight of the cell.

#### *Protein estimation*

Protein estimation was carried out according to the method of Lowry *et al.*, (1951).

#### *Chemicals*

[1,2, <sup>14</sup>C]-acetate and <sup>45</sup>Ca in HCl (5 mCi) were obtained from the Bhabha Atomic Research Centre, Trombay, Bombay. All the standard lipids for TLC, PPO, POPOP,

ATP, CoA, NAD, dithionitro-benzoic acid, HEPES, dipicolinic acid, tris (hydroxy methyl) aminomethane, bovine serum albumin, DL-threonine, hydroxylamine, azoalbumin and the substrates for the enzyme assays were obtained from Sigma Chemical Co., St. Louis, Missouri, U.S.A. L-Cystine was obtained from E. Merck, Darmstadt, Germany. All the other chemicals used were of the reagent grade.

## Results

### *Effect of cystine on calcium uptake*

In the control medium, the uptake of  $[^{45}\text{Ca}^{2+}]$  increased gradually and reached a maximum at 5-6 h of the stationary phase. In the experimental cells calcium uptake was inhibited (table 1).

**Table 1.** Effect of cys/cysSH on various macromolecular events in *B. thuringiensis*

Macromolecular events	Maximum value obtained after the stationary phase		Percentage inhibition in the experimental
	Control	Experimental	
Calcium uptake ( $\text{cpm} \times 10^{-4}/\text{mg protein}$ )	6	0.2	97
Dipicolinic acid synthesised ( $\mu\text{g}$ dipicolinic acid/10ml culture)	550	0.5	100
Enzymes of dipicolinic acid synthesis (units/mg protein):			
a) Aspartokinase	14	0	100
b) Dihydrodipicolinic acid synthase	8	0	100
Metalloprotease (units/ml culture)	125	0	100
Enzymes of Krebs cycle (units/mg protein);			
a) Pyruvate dehydrogenase	3	3	0
b) Citrate synthase	3	3	0
c) Aconitase	2	0	100
d) Isocitrate dehydrogenase	3	0	100
e) $\alpha$ -Ketoglutarate dehydrogenase	0.01	0.01	0
f) Succinic dehydrogenase	2.8	0	100
g) Fumarase	18	0	100
h) Malic dehydrogenase	0.6	0	100
Acetate uptake ( $\text{cpm} \times 10^{-4}/\text{mg dry wt}$ )	8	0.2	99
Lipid synthesis ( $\text{cpm}/\text{mg dry wt}$ )	8041	66	99

### *Dipicolinic acid synthesis*

Synthesis and accumulation of dipicolinic acid increased gradually in the normal sporulating cell and reached a maximum value after 4-5 h of the stationary phase (table 1). From this time onwards, the spore could be visualized under a phase contrast microscope. Dipicolinic acid could not be detected in the experimental cultures (table 1).

*Effect of cystine on the enzymes of dipicolinic acid synthesis*

Very low activity (1 unit per mg protein) of aspartokinase was present in the vegetative cells of *B. thuringiensis* var. *thuringiensis*. The activity was found to increase 90 min after the cells had reached the stationary phase and the aspartokinase activity reached a maximum value at 6 h of the stationary phase. In the experimental cells, very little activity of aspartokinase was detected in the vegetative cells; thirty min after the onset of the stationary phase the enzyme activity was not detected (table 1). A very low activity of dihydropicolic acid-synthase could be detected in the control during the early stationary phase which increased gradually and reached a maximum value at 8-9 h of the stationary phase. In the experimental cells, there was gradual decrease in the activity from 1 unit per mg protein present at 0 h to zero after 2 h of the stationary phase (table 1).

*Effect of cystine on metalloprotease production*

Four to five h after the cessation of vegetative growth, the metalloprotease activity increased rapidly from the basal level and reached a maximum of 125 units per ml in the control. The protease activity reached a peak at about the same time as the appearance of heat stable spores and complete crystals. In the experimental culture, very little of metalloprotease activity was detected in the medium during the early stationary phase which declined within 2-3 h of the stationary phase (table 1). The addition of  $Mg^{2+}$  or  $Mn^{2+}$  did not produce any effect on the metalloprotease production either in the control or in the experimental culture.

*Effect of cystine on the enzymes of Krebs' cycle*

Enzymes of the Krebs' cycle were found to be stimulated 2-3 fold after the cessation of the vegetative growth. Such a stimulation was observed only in the control cells. Irrespective of the cystine concentration, the activities of pyruvate dehydrogenase and citrate synthase were the same in the control and experimental cells. Considerably very low activity of  $\alpha$ -ketoglutarate dehydrogenase was detected both in the control and experimental cells. All other enzymes of the Krebs' cycle i.e., from aconitase onwards were found to be repressed in the experimental cells (table 1).

*Lipid synthesis in relation to cystine concentration*

There was a slight increase in the lipid synthesis in the control. After the stationary phase, the incorporation of [ $^{14}C$ ]-acetate into the phospholipid was more than that into the neutral lipid, in the control. In the experimental cells, the incorporation of [ $^{14}C$ ]-acetate into the lipid moiety was observed only in the mid log phase (when the carbon source, glucose was removed from the medium). Acetate incorporation into the lipid was almost negligible in the experimental cells, from 1 h before the cessation of vegetative growth (table 1).

*Uptake of [1,2, $^{14}C$ ]-acetate in relation to cystine concentration:*

Control cells take up (1,2, $^{14}C$ )-acetate from 4 h prior to the stationary phase. This uptake increased gradually and reached a maximum value at 2 to 3 h of the stationary phase. Acetate incorporation was completely inhibited in the experimental cells (table 1).

## Discussion

During the transition from the logarithmic to the stationary phase, in the sporulating bacilli, various macromolecular events take place. Several catabolic enzymes and secondary metabolites were implicated in sporogenesis since they appear shortly after the end of exponential growth (Hanson *et al.*, 1964; Hanson *et al.*, 1961; Lang and Lundgren, 1970; Li and Yousten, 1975; Powell, 1953; Powel and Strange, 1956). These include the uptake of certain metal ions (Vinter, 1969), derepression of the activities of some of the membrane-bound enzymes (Bulla *et al.*, 1971), synthesis of dihydrodipicolinic acid (Hoganson and Stahly, 1975; Powell, 1953), production of antibiotics (Bernlohr and Novelli, 1963), synthesis of lytic enzymes (Powell and Strange, 1956), production of toxins (Bulla *et al.*, 1977; 1979; Schaeffer, 1969) and the appearance of certain specific products like protease and antigens (Baillie and Norris, 1964). There are no reports to specify whether these changes take place in *B. thuringiensis* var. *thuringiensis* and whether the reactions occurring during sporulation are related to the crystal formation also.

A decrease in the ability to sporulate also brings about decreased calcium uptake (as seen in table 1). In addition to calcium uptake, dipicolinic acid synthesis was found to be inhibited in the non-sporulating culture. Dipicolinic acid is an essential component of the spore (Forman and Aronson, 1972). It is not clear how  $\text{Ca}^{2+}$  deprivation inhibits dipicolinic acid synthesis. The observation that the activities of both aspartokinase and dihydrodipicolinic acid synthase were blocked in the experimental cells during the early stationary phase showed that the dipicolinic acid synthesis was inhibited at the early stages of its synthetic pathway. This could account for the 'shunt' in the spore and crystal formation. Besides, the fact that the activities of aspartokinase and dihydrodipicolinic acid synthase were normal in the vegetative cell shows that Cys would not have produced any effect in the vegetative growth phase, but only during the sporulation phase. Comparative studies on the production of metalloprotease in control and experimental cells showed a marked difference in the enzyme level. The observed inhibition of protease production in the experimental cells might be one of the reasons for the inhibition of spore and crystal formation in *B. thuringiensis* var. *thuringiensis*.

The relationship between the tricarboxylic acid cycle and sporulation was first detected in a mutant of *B. subtilis* lacking aconitase (Hanson *et al.*, 1964). The tricarboxylic acid cycle enzymes were repressed during the vegetative growth of several species of spore forming bacilli, in a complex medium containing glucose (Hanson *et al.*, 1961). These enzymes were derepressed after the exponential phase. The lack of a fully operational tricarboxylic acid cycle was reported in *B. thuringiensis* (Bulla *et al.*, 1970). It was proposed that this cycle is modified in *B. thuringiensis* where the  $\alpha$ -ketoglutarate is converted to succinate via glutamate and  $\gamma$ -amino-butyric acid (Aronson *et al.*, 1975). High levels of glutamate decarboxylase activity were detected in *B. thuringiensis* (Aronson *et al.*, 1975). Nickerson *et al.* (1974) had shown that *B. thuringiensis* sporulates in a glucose-glutamate medium without the concurrent derepression of the tricarboxylic acid cycle; without glutamate no sporulation was observed. The observed inhibition of aconitase which has been implicated as a key enzyme functioning during sporulation (Hanson *et al.*, 1961) could have resulted in the inhibition of spore crystal formation in *B. thuringiensis* var. *thuringiensis*.

One of the first visible events in bacterial sporogenesis was the development of an asymmetric spore membrane septum followed by its extension to form the forespore membrane (Ellar and Lundgren, 1966). In *B. megaterium* and *B. cereus*, the levels of phospholipid were found to depend on the age of the culture (Bertsch *et al.*, 1969; Lang and Lundgren, 1970). In the present study the lipid composition of *B. thuringiensis* var. *thuringiensis* was found to be similar to that of other bacilli (Bulla *et al.*, 1970). Changes in the phospholipid content were found to occur in the control and in the experimental cells respectively after the stationary phase. Lipid synthesis was also found to be inhibited in the experimental cells. Such a disturbance in the lipid synthesis could have led to the inability of spore membrane formation and in turn spore and crystal formation in *B. thuringiensis* var. *thuringiensis*.

Acetate uptake and metabolism were found to be essential steps for sporulation (Hanson *et al.*, 1961). In the experimental cells acetate uptake was negligible compared to the control. It was already suggested that cystine may be important in regulating the membrane synthesis (Bulla *et al.*, 1977). The presence of excess cystine might not have been able to perform such a regulation in *B. thuringiensis* var. *thuringiensis*. Very little is known about the inhibition of sporulation in *B. megaterium* by the addition of Cys/CysSH (Vinter, 1951). Apart from this there are no reports on the aspect of inhibition of the sporulation by Cys/CysSH. Since most of the sporulation-specific events have been found to be affected in the experimental cells, it appears that excess cystine may have interfered with some of the events of spore and crystal formation in *B. thuringiensis* var. *thuringiensis*. It also shows that the sporulation-specific events could be necessary for the crystal formation also.

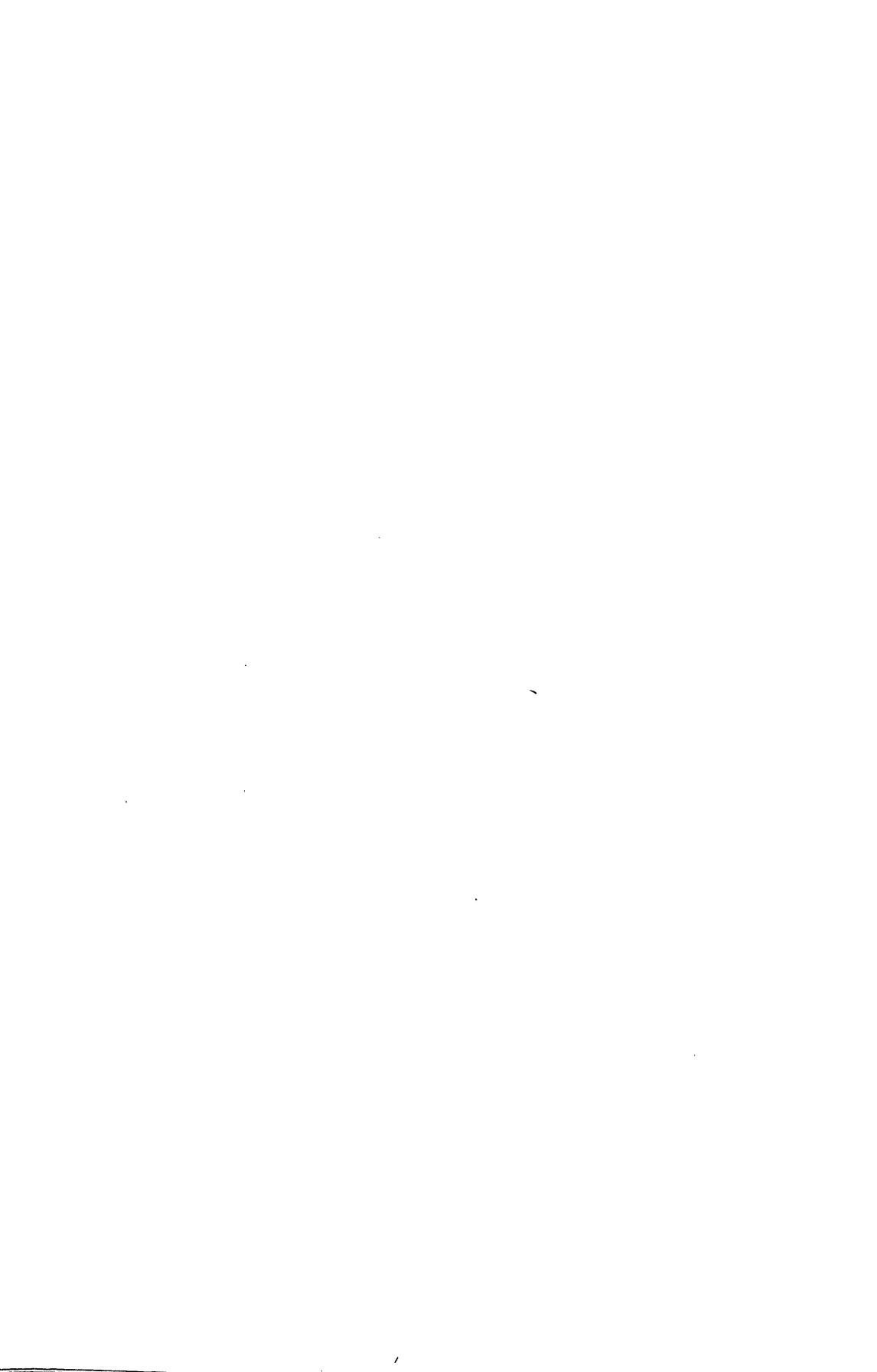
### Acknowledgement

We thank Dr. M. S. Shaila for many useful discussions.

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## Spore and crystal formation in *Bacillus thuringiensis* var. *thuringiensis* during growth in cystine and cysteine.

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MS received 24 July 1980; revised 22 October 1980.

**Abstract.** The effect of the addition of different concentrations of cystine and cysteine on sporulation and parasporal crystal formation in *Bacillus thuringiensis* var. *thuringiensis* was studied. The effect was well pronounced when the cystine/cysteine additions were made after the stationary phase. Heat stable spores and crystals were formed when the culture was provided with a low concentration of cystine/cysteine (0.05 per cent w/v). At a moderate concentration of cystine or cysteine (0.15%), only heat labile spores were formed without the production of the crystal. When the cystine/cysteine concentration was high (0.25%), spore and crystal formation were completely inhibited. Partial reversal of inhibition of sporulation was brought about by sodium sulphate or zinc sulphate and lead, copper, cadmium or cobalt acetate at 0.2 mM or at 0.2% of sodium or potassium pyruvate, citrate, cisaconitate, oxalosuccinate,  $\alpha$ -keto-glutarate, succinate, fumarate, malate, or oxalacetate. Glutamate (0.2%) overcame the inhibitory effect of cystine/cysteine completely. The structural changes observed using phase contrast microscopy were dependent upon the concentration of cystine/cysteine.

**Keywords.** *Bacillus thuringiensis* var. *thuringiensis*. cystine, cysteine, spore, crystal, reversal of inhibition.

### Introduction

*Bacillus thuringiensis*, an important insecticide (Bulla *et al.*, 1975) is toxic mainly to lepidopteran larvae (Rogoff and Yousten, 1969). The toxicity resides in an intracellular parasporal crystalline inclusion (Angus, 1954, 1956a, 1956b, 1956c; Rogoff and Yousten, 1969; Heimpel and Angus 1960; Nickerson *et al.*, 1974). Upon ingestion of the proteinaceous crystal by susceptible larvae, proteolytic enzymes in the larval gut juice hydrolyze the crystal protein into as yet undefined toxic moieties. This is followed by paralysis and eventual death of the larvae (Angus, 1954; Angus, 1956a; Angus and Heimpel, 1959).

Since sporulation and germination in bacilli are dependent on the nutritional status of the organism (Hardwick and Foster, 1952), a study of the nutritional requirement of *Bacillus thuringiensis* var. *thuringiensis* is important for delineating the control mechanisms which regulate spore and parasporal crystal formation. Certain amino acids support growth, sporulation and crystal formation of *B. thuringiensis*

var. *thuringiensis*, while others inhibit the growth (Singer *et al.*, 1966; Singer and Rogoff, 1968; Bulla *et al.*, 1975; Nickerson and Bulla, 1975; Rajalakshmi and Shethna, 1977). A lower concentration of cystine (Nickerson and Bulla, 1975) or cysteine (Rajalakshmi and Shethna, 1977) promotes growth, sporulation and crystal formation in *B. thuringiensis*, while at a higher concentration of cys/cysSH, only the vegetative growth was observed, (Rajalakshmi and Shethna, 1977).

We report here a detailed study of the effect of various concentrations as well as the time of addition of cys/cysSH on spore and crystal formation in *B. thuringiensis* var. *thuringiensis* and the reversal of inhibition of sporulation by various compounds.

## Materials and methods

### *Organism and culture conditions*

*Bacillus thuringiensis* Serotype I was obtained from Professor H. de Barjac, Institute Pasteur, Paris, France. The culture was maintained on nutrient agar slants. The minimal medium and the inoculum were prepared as described earlier (Rajalakshmi and Shethna, 1977).

### *Effect of cys/cysSH*

To study the effect of various concentrations of cys/cysSH the minimal medium containing 1% glucose was supplemented with 0.05, 0.1, 0.15, 0.2 and 0.25% of cys/cysSH, respectively. Growth, sporulation and crystal formation were monitored from time to time.

### *Effect of addition of cys/cysSH after the stationary phase*

In another set of experiments, *B. thuringiensis* var. *thuringiensis* cells were grown in a minimal medium containing 1.0% glucose and 0.05% cys/cysSH. Different concentrations of cys/cysSH (i.e. 0.05, 0.1, 0.15 and 0.2%) were added at 0, 1, 2, 3 and 4 h after the stationary phase was reached and their effects on spore and crystal formation were studied.

### *Microscopic studies*

For phase contrast microscopy, control and experimental cells were harvested at the mid log phase and 10 h after the attainment of the stationary phase. The cells were washed and suspended in 0.1 M potassium phosphate buffer pH 7.2. A wet smear of the suspension was observed immediately under a light microscope (Zeiss photo microscope, W. Germany).

### *Reversal of inhibition of sporulation*

A reversal of inhibition of sporulation, caused by cys/cysSH was effected by the addition of divalent cations, intermediates of the tricarboxylic acid cycle or glutamate individually. Sodium or zinc sulphate and lead, copper, cadmium and cobalt acetate were used (2 mM). Sodium or potassium purvate, citrate, *cis*-aconitate, oxalosuccinate  $\alpha$ -keto-glutarate, succinate, fumarate, malate, oxalacetate and glutamate were used at a concentration of 0.2% after filter sterilization.

Additions were made 60 to 90 min prior to the stationary phase, when varying concentration of cys/cysSH was provided in the growth medium. Whereas, when

the cys/cysSH additions were made after the stationary phase, the substances were added 10 to 15 min prior to the addition of excess cys/cysSH.

Cys and cysSH, wherever mentioned, were used individually. Growth was monitored by measuring the absorbance at 600 nm in a Bausch and Lomb Spectronic-20 colorimeter. Sporulation and crystal formation were monitored by the observation of a wet smear of the culture under a phase contrast microscope (Zeiss dark phase contrast microscope). Heat-stable spores were estimated by a standard procedure (Nickerson and Bulla, 1975). When the cells were grown with low concentration of cys/cysSH (0.05%), heat-stable spores and crystals were produced; under such a condition, there was no heat-labile spore formation. When the cells were provided with a moderate concentration of cys/cysSH (0.15%), only heat labile spores were produced. The heat-labile spores were found to be susceptible to lysozyme treatment, whereas the heat-stable spores were not susceptible to such treatment. The heat-labile spores were estimated by plating on nutrient agar plates after appropriate dilution. Heat lability and stability were confirmed by lysozyme treatment (Stahly *et al.*, 1978). Cells grown 0.05% cys/cysSH and 1% glucose were taken as the control for all the studies given under Materials and Methods. Toxicity of the crude cell lysate was checked by feeding the pellet obtained after centrifugation of the crude cell lysate at 8,000 g for 10 min, to the second or third instar larvae of silkworm moth (*Bombyx mori*). The crude pellet was suspended in distilled water, sprayed on mulberry leaf (1 mg protein) and fed to 20 larvae.

### Chemicals

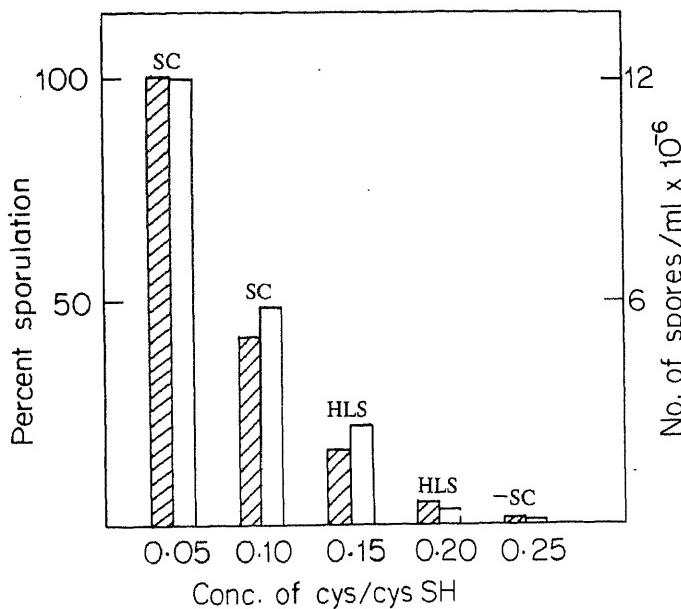
Intermediates of tricarboxylic acid cycle and glutamate were obtained from Sigma Chemical Co., St. Louis, Missouri, USA. L-Cystine, L-cysteine\* and all the salts of divalent cations were obtained from E. Merck, Darmstadt, Germany. All the other chemicals were of Analar or Reagent grade.

## Results

### *Spore and crystal formation in relation to cys/cysSH concentration*

The efficiency of spore and crystal formation was studied in relation to cys/cysSH concentration. It was noticed that in the control, as well as in the presence of cys/cysSH (0.05%), the efficiency of spore and crystal formation was 100 per cent. At a concentration of 0.1% of cys/cysSH, the efficiency was drastically reduced. At 0.15 and 0.2% of cys/cysSH, only heat-labile spores were formed with complete inhibition of crystal formation. At 0.25% cys/cysSH, there was complete inhibition of spore and crystal formation in *B. thuringiensis* var. *thuringiensis* (figure 1).

\* Cysteine was freely soluble upto 0.112g/1 at 25°C. Whenever needed, it was solubilized with acid (0.1 N HCl) and then added to the medium. The pH of the medium was adjusted to 7.2 after the cys/cysSH addition. (Reference: The Merck index of chemicals and drugs, 7th ed. Merck and Co. inc., Rahway, New Jersey, USA 1960.)



**Figure 1.** Effect of cys/cysSH on spore and crystal formation in *B. thuringiensis* var. *thuringiensis*. Heat-labile and heat-stable spores were calculated as given under Materials and Methods. Toxicity was checked by feeding to 2nd or 3rd instar silkworm (*Bombyx mori*) as mentioned in the text. SC-Heat stable spores and crystals. HLS-Heat labile spores. -SC: No spore and crystal. Since the absorbance increased with respect to cys/cysSH concentration, all the samples were diluted so as to obtain an absorbance equal to control (0.05 per cent cys/cysSH). After dilution, the number of spores per ml was estimated. Cystine, (■); Cysteine, (□).

#### Effect of addition of cys/cysSH at the stationary phase

It was noticed that when 0.05% of cys/cysSH was added at 0 h of the stationary phase, heat-stable spores and toxic crystals were formed. At 0.1 and 0.15%, only heat-labile spores were produced with no crystal formation. Where as, when 0.2% of cys/cysSH was added, spore and crystals were not formed (table 1) microscopically, and the bioassay also showed no toxicity.

#### Effect of addition of cys/cysSH after the stationary phase

Sporulation and parasporal crystal formation were found to be inhibited even at the concentration of 0.15% of cys/cysSH when the addition was made 1 h after the onset of the stationary phase (table 1). The efficiency of sporulation decreased as the time of addition of excess cys/cysSH was increased.

Per cent	No. of Spores/ml				
	t <sub>0</sub>	t <sub>1</sub>	t <sub>2</sub>	t <sub>3</sub>	t <sub>4</sub>
Cys/cysSH					
0.05	12.7×10 <sup>6</sup>	5.3×10 <sup>4</sup>	2×10 <sup>2</sup>	12	—
0.1	5.8×10 <sup>4</sup>	1.5×10 <sup>2</sup>	6×10 <sup>1</sup>	4.6	—
0.15	2.0×10 <sup>2</sup>	4.5	—	—	—
0.2	—	—	—	—	—
CysSH					
0.05	12.5×10 <sup>6</sup>	4.9×10 <sup>4</sup>	2.7×10 <sup>2</sup>	18	—
0.1	5.6×10 <sup>4</sup>	2.8×10 <sup>2</sup>	19×10 <sup>1</sup>	6.8	—
0.15	1.5×10 <sup>2</sup>	11-12	—	—	—
0.2	—	—	—	—	—

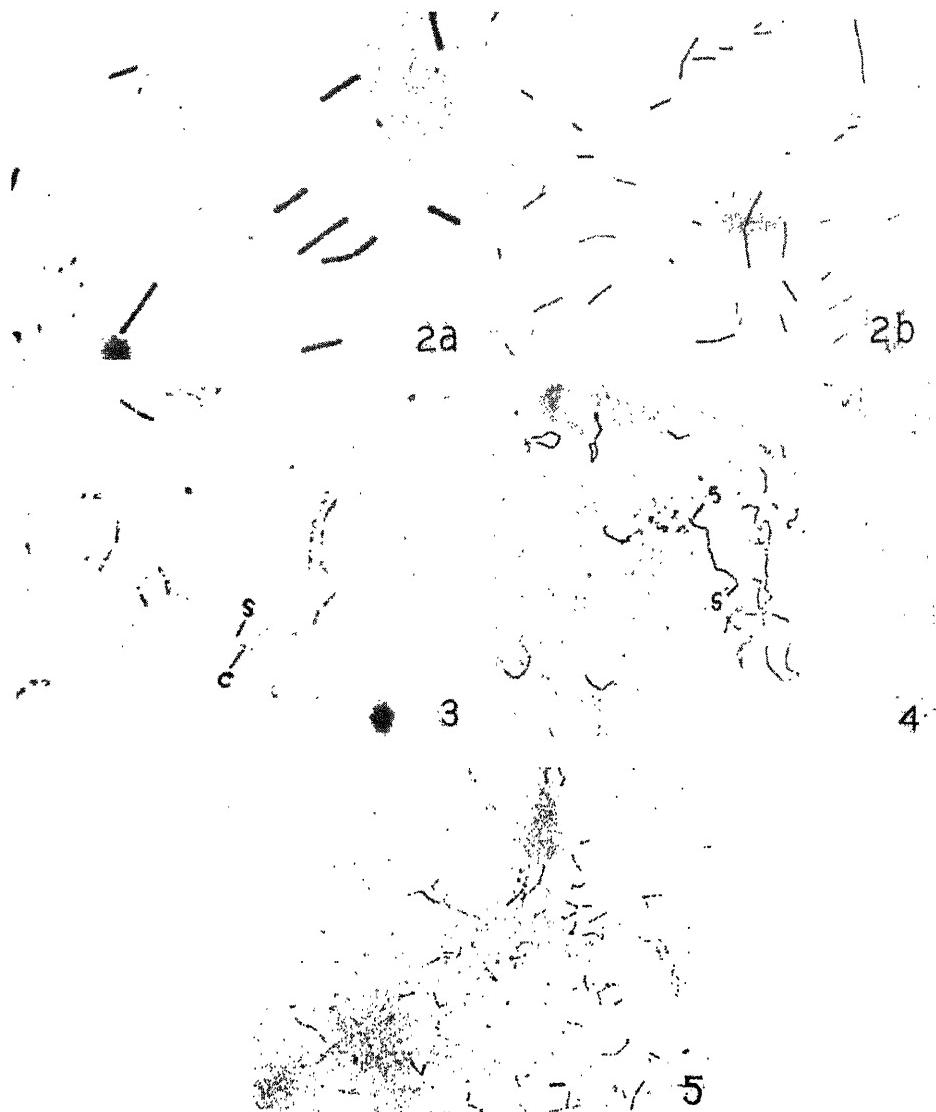
**Table 1.** Effect of addition of cys/cysSH after stationary phase.

O, t<sub>0</sub>, t<sub>1</sub>, t<sub>2</sub>, t<sub>3</sub>, t<sub>4</sub> represent the time of addition of cys/cysSH i.e., O h—at stationary phase; t<sub>1</sub>—1 h after stationary phase; t<sub>2</sub>—2 h after stationary phase etc. 10-12 h after the addition of cys/cysSH samples were removed and the number of spores were calculated. Toxicity was checked as per the text. Only when 0.05% cys/cysSH was added at t<sub>0</sub> and t<sub>1</sub>, there was the formation of the heat stable spores and crystals.

When 0.1% cys/cysSH was added after t<sub>2</sub> and 0.15% cys/cysSH was added even at t<sub>0</sub> only heat labile spores were produced without the crystal formation. For this addition experiment cells were grown with 1% glucose and 0.05% cys/cysSH.

### Microscopy

The cells grown on cys/cysSH medium were thinner and longer than the control cells (figures 2a, 2b). Photomicrography revealed that the control cells grown with 0.05% cys/cysSH produced spores and crystals (figure 3). At moderate concentration of cys/cysSH (0.15%) only heat-labile spores were formed (figure 4). At high concentration (0.25%), both spore and crystal formation were found to be completely inhibited; besides, there was a change in the morphology of the experimental cells, about 15 to 29 min before lysis (figure 5).



**Figure 2.** Photomicrograph of the control (2a), and the experimental (2b) cells at the late logarithmic phase. (magnification  $\times 1800$ ).

**Figure 3.** Photomicrograph of the control cells 12 h after the stationary phase. S—Spore; C—Crystal. (magnification  $\times 1400$ ).

**Figure 4.** Photomicrograph of the cells grown with 0.15% cys/cysSH. Micrograph taken 12 h after the stationary phase. S—Heat labile spore. (magnification  $\times 1400$ ).

**Figure 5.** Photomicrograph of the cells grown with 0.25% cystine. Picture taken at 15 min before the lysis of the cells i.e., 8 h after the stationary phase. (magnification  $\times 1400$ ).

*Reversal of inhibition of sporulation caused by cys/cysSH*

Partial reversal of the inhibition of sporulation was observed on the addition of certain divalent cations as well as some of the intermediates of Krebs' cycle (figures 6, 7). In addition, glutamate (0.2%) also brought about complete reversal of inhibition of sporulation (figure 7). Heat-stable spores and crystals were formed whenever the reversal of inhibition was observed.

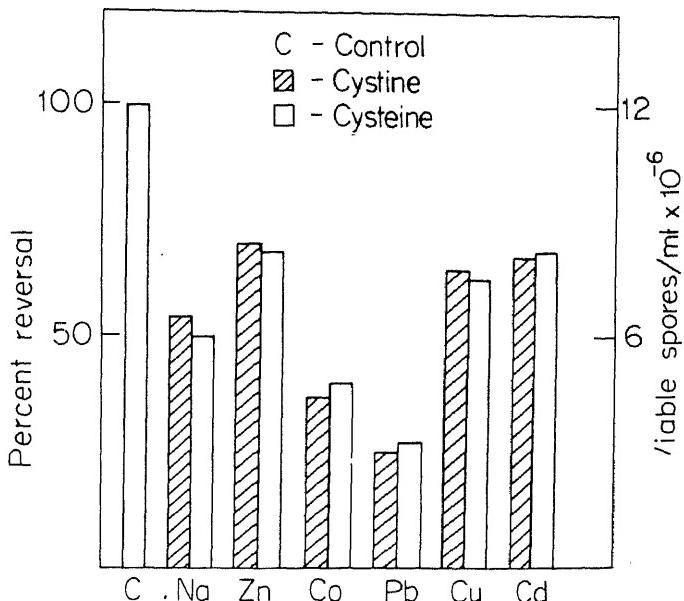


Figure 6. Reversal of inhibition of sporulation caused by cys/cysSH by the addition of divalent cations. C—control grown with 1% glucose and 0.05% cys/cysSH; Na—sodium sulphate; Zn—Zinc sulphate; Co—Cobalt acetate; Pb—Lead acetate; Cu—Copper acetate; Cd—Cadmium acetate.

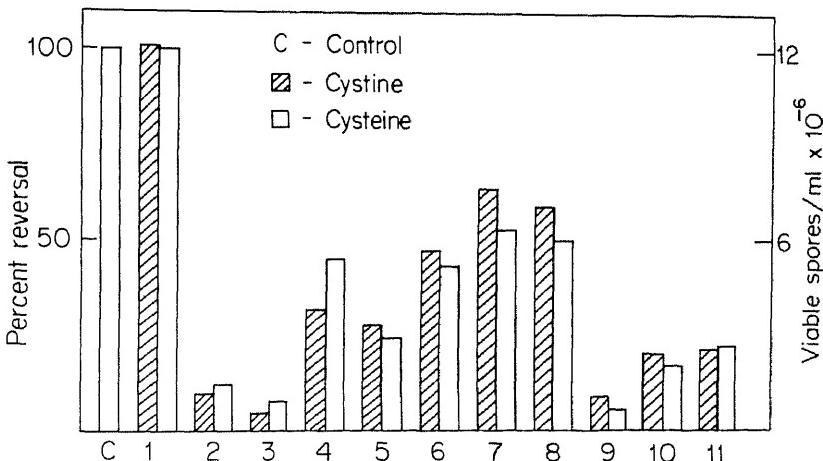


Figure 7. Reversal of inhibition by Krebs cycle intermediates and glutamate. C—Control; 1—pyruvate; 2—citrate; 3—*cis*-aconitate; 4—isocitrate; 5—oxaloacetate; 6—ketoglutarate; 7—succinate; 8—fumarate; 9—malate; 10—oxaloacetate; 11—glutamate. Heat-stable spores and crystals were produced whenever the phenomenon of reversal was observed.

### **Discussion**

The reversal of inhibition caused by cys/cysSH on the addition of heavy metal salts could be occurring through a mechanism similar to that proposed for *B. megaterium* (Vinter, 1957). It was presumed that heavy metal ions reacted with H<sub>2</sub>S produced from cystine by the organism and hence led to the reversal of the inhibition of the sporulation. It is possible that cys/cysSH breakdown and incorporation were intensified by increased respiration (Vinter, 1957) in presence of intermediates of the tricarboxylic acid cycle leading to reversal of inhibition.

Glutamate plays an important role during sporulation by acting as the primary energy source for the assimilation of acetate (Nakata and Halvorson, 1960) into parahydroxybutyrate (Charba and Nakata, 1977; Kennedy *et al.*, 1971). The ability of glutamate to reverse the inhibition of sporulation and crystal formation could be due to an increased respiration, the induction of enzymes of the γ-amino butyric acid pathway, the incorporation and assimilation of acetate or the cumulative effect of all the three.

Changes observed at the structural level (figures 2b, 5) could be due to a disturbance at the physiological level caused by excess cys/cysSH in the medium. Growth was enhanced as the concentration of cys/cysSH was increased in the medium, whereas only the spore and crystal formation were affected. The observed inhibition of sporulation could not be due to acidity of the medium caused by cys/cysSH as the pH was maintained at 7.2 and the free amino acids rather than the hydrochlorides were used. The identical results obtained with cys and cysSH could be due to the fact that cysteine is first converted to cystine and then metabolized further.

### **Acknowledgement**

We thank Dr. M. S. Shaila for many useful discussions and help in the preparation of the manuscript.

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## Intra and inter generic homology in polysaccharide structure of *Rhizobium* and *Alcaligenes*.

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MS received 19 July 1980; revised 4 September 1980

**Abstract.** A study was conducted on the structure of extracellular, water-soluble polysaccharides from 5 different strains of *Rhizobium* viz. *R. trifolii* J60 and *R. meliloti* strains J7017, 202, 204 and 207. All these polysaccharides were found to contain glucose and galactose in the approximate molar ratio of 7:1. Methylation analysis revealed these polysaccharides to contain (1 → 3), (1 → 6), (1 → 4), (1 → 4, 1 → 6)-linked D-glucose residues, (1 → 3)-linked D-galactose and non-reducing terminal D-glucose attached to pyruvate. These polysaccharides were also found to be acylated by both acetyl and succinyl residue. This structure was found to be similar to that of succinoglycan, a succinic acid-containing water-soluble, extra-cellular polysaccharide elaborated by *Alcaligenes faecalis* var. *myxogenes* 10C3. This similarity in structure of polysaccharides from two different species of *Rhizobium* and also the polysaccharide produced by *Alcaligenes* has been discussed.

**Keywords.** *Rhizobium*, *Alcaligenes*, polysaccharides, structure, homology, methylation analysis.

### Introduction

Symbiotic bacteria belonging to the genus *Rhizobium* play an important role in the nutrition of leguminous plants by fixing atmospheric nitrogen in the root nodules. The ability to form nodules has been found to be highly host specific for different *Rhizobium* species (Fahraeus and Ljunggren, 1968). Some reports suggest the involvement of bacterial capsular polysaccharides in the Rhizobial host specificity (Fahraeus and Ljunggren, 1968; Bjorndal *et al.*, 1971). Hence the structural analysis of these polysaccharides is a matter of interest.

### Materials and methods

#### *Strains*

*R. trifolii* J60 was obtained from Prof. Y. Maruyama of the University of Tokyo, and *R. meliloti* strains J7017, 204, 202 and 207 were obtained from Prof. M. Yatazawa of Nagoya University, Nagoya, Japan.

#### *Production of polysaccharides*

Succinoglycan, an acidic, water-soluble polysaccharide elaborated by *Alcaligenes faecalis* var. *myxogenes* 10C3 was supplied by Dr. A. Amemura of Osaka University,

Osaka, Japan. *Rhizobium* polysaccharides were produced in a chemically defined medium (Amemura and Harada, 1971) supplemented with 0.1% yeast extract, contained in 500 ml Erlenmeyer flasks (each containing 100 ml medium) and incubating for 7 days at 30°C on a gyratory shaker.

The water soluble polysaccharides were separated from the culture medium by centrifugation and precipitation with acetone and cetylpyridinium chloride as described by Misaki *et al.* (1969). The dried polysaccharides were redissolved in water (to 0.1% concentration), dialyzed for two days against distilled water and freeze-dried.

#### *Quantitative analysis of sugars*

Polysaccharides were hydrolyzed, individually, in a sealed tube with 1 ml of 90% formic acid for 6 h at 100°C. The hydrolysate was converted to alditol acetates and analyzed (Björndal *et al.*, 1967) in a Shimadzu GC7A gas chromatograph, fitted with a flame ionization detector and a column (4mm × 200 cm) of 3% ECNSS-M on Gas-chrom Q at 190°C. Xylose was used as an internal standard.

#### *Quantitative analysis of organic acids*

Pyruvic acid in water-soluble polysaccharides was assayed by the method of Koepsell and Sharpe (1952). Acids in the form of esters were assayed by the colorimetric method of McComb and McCready (1957). Succinic acid and acetic acid were assayed by high speed liquid chromatography as described by Hisamatsu *et al.* (1978).

#### *Optical rotation*

Optical rotation was measured in a Perkin-Elmer polarimeter using a 0.25% polysaccharide solution in water.

#### *Preparation of deacylated polysaccharides*

A 0.1% solution of the polysaccharide was stirred in 100 mM KOH at 20°C followed by dialysis and freeze drying as suggested by Sloneker and Jeanes (1962).

#### *Methylation analysis of polysaccharides*

Methylation of native and depyruvylated (prepared by the method of Chaudhari *et al.*, 1973) polysaccharides was done by the Hakomori (1964) technique followed by gas chromatography using an OV 275-GEX 1150 column. The overall method and scheme for methylation was essentially that reported by Hisamatsu *et al.* (1980).

### **Results and discussion**

A study was carried out on the extra-cellular, water-soluble polysaccharides of 13 different strains belonging to three species of *Rhizobium*. Out of these, polysaccharides of 5 strains viz. *R. trifoli* J60, *R. meliloti* strains J7017, 202, 204 and 207 showed similar structure and are reported.

*R. trifoli* J60 and *R. meliloti* strains J7017, 202, 204 and 207 when grown in the chemically defined medium of Amemura and Harada (1971) supplemented with 0.1% yeast extract gave 580, 220, 330, 355 and 340 mg yields of polysaccharides respectively per 100 ml medium.

*Components of water-soluble polysaccharides*

The composition of all the 5 *Rhizobium* polysaccharides (Table 1) was studied. All the polysaccharides were found to contain glucose and galactose (Figure 1) in an approximate ratio of 7:1. Humphrey and Vincent (1959) and Zevenhuizen (1973) have reported the presence of uronic acids in *Rhizobium* polysaccharides but in this study uronic acids were not detected in any of the 5 polysaccharides. Pyruvate content was 5.5-11.2% in the polysaccharides. Pyruvate had already been reported to be a constituent of *Rhizobium* polysaccharides by Björndal *et al.*, (1971), Zevenhuizen (1971, 1973), Chaudhari *et al.* (1973) and Dudman (1976). Acids in the form of esters were assayed by the method of McComb and McCready (1957) and ranged between 1.9 to 3.7%. Identification by high speed liquid chromatography, revealed the presence of succinate and acetate in all the 5 polysaccharides. The presence of acetate, but not succinate, has also been reported previously (Zevenhuizen, 1973) in *Rhizobium* polysaccharides.

**Table 1.** Composition and optical rotation of *Rhizobium* polysaccharides and succinoglycan.

Composition (%)	Organism					Succinoglycan	
	<i>R. trifolii</i>		<i>R. meliloti</i>				
	J60	J7017	202	204	207		
Glucose	78.2	77.2	79.6	78.8	78.1	78.0	
Galactose	11.5	11.0	11.6	11.4	11.4	10.1	
Pyruvate	11.2	9.8	5.5	6.2	6.3	5.4	
O-acetyl <sup>a</sup>	1.9	3.7	3.2	3.1	2.9	3.4	
Acetate <sup>b</sup>	0.6	2.2	1.8	1.3	1.5	0	
Succinate	1.3	1.8	1.5	1.8	1.4	3.3	
$[\alpha]_{D}^{25}$	-23	-21	-19	-13	-26	-15	

All values have been expressed as % except for  $[\alpha]_{D}^{25}$

<sup>a</sup> Estimated colorimetrically.

<sup>b</sup> Estimated by high speed liquid chromatography.

The optical rotation,  $[\alpha]_{D}^{25}$  of the polysaccharides ranged from -13 to -26. The negative values suggested that the sugar constituents of the polysaccharides were predominantly linked by  $\beta$ -linkages.

*Methylation analysis of the water-soluble polysaccharides*

Methylation of the polysaccharides was done by the Hakomori (1964) technique to find out the type of linkages between various sugar components. Alditol acetates of methylated native and depyruvylated polysaccharides were separated by gas chromatography on an OV 275-GEX 1150 column. The conditions and detailed procedure of the use of this column for methylation analysis have been reported by Hisamatsu *et al.* (1980).

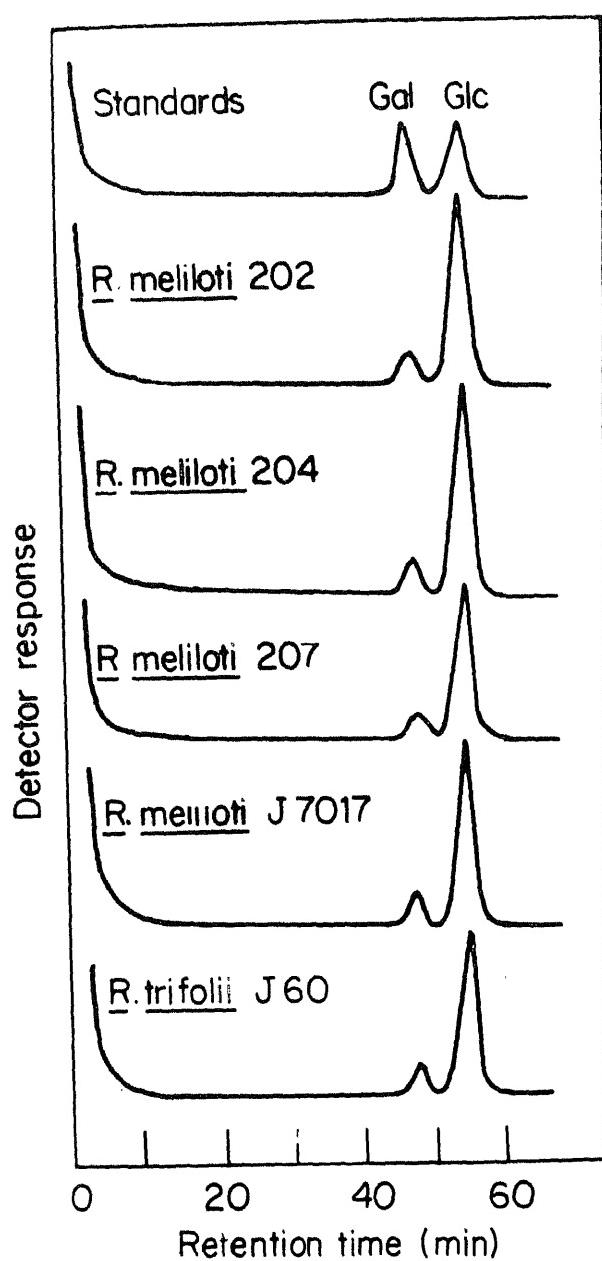
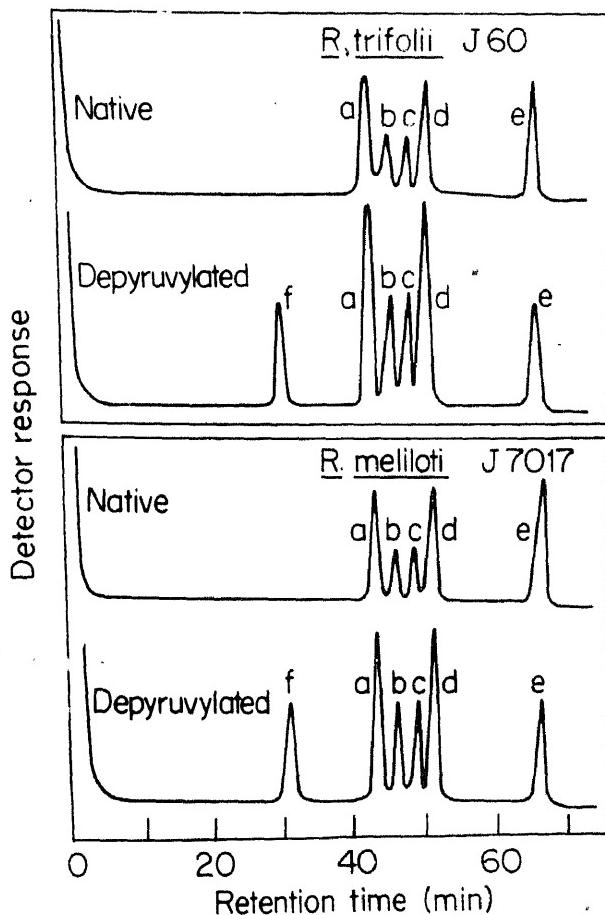


Figure 1. Gas liquid chromatographic patterns of alditol acetates of *Rhizobium* polysaccharides on an ECNSS-M column.

Figure 2 gives the gas chromatogram profiles of methylated native and depyruvated polysaccharides of *R. trifolii* J60 and *R. meliloti* J7017. The gas chromatographic patterns of *R. meliloti* strains 202, 204 and 207 were also the same and the quantitative formation is given in Table 2.



**Figure 2.** Gas liquid chromatographic patterns of alditol acetates of methylated native and depyruvylated polysaccharides on an OV 275-GEX column. (A) native (B) depyruvylated *Rhizobium trifolii* J60 and *Rhizobium meliloti* J7017 polysaccharides.

(a) 2, 4, 6-tri-*O*-methyl-D-glucitol, (b) 2, 4, 6-tri-*O*-methyl-D-galactitol, (c) 2, 3, 4-tri-*O*-methyl-D-glucitol, (d) 2, 3, 6-tri-*O*-methyl-D-glucitol, (e) 2, 3-di-*O*-methyl-D-glucitol and (f) 2, 3, 4, 6-tetra-*O*-methyl-D-glucitol.

Table 2. Methylation analysis of succinoglycan (SG) and *Rhizobium* polysaccharides.

Methoxylated sugar (Relative proportion)	Polysaccharides (native depyruvylated)					
	<i>R. trifoli</i>			<i>R. meliloti</i>		
	SG	J60	J7017	202	204	207
2, 3, 4, 6-Glc (terminal)	0(1.0)	0(1.0)	0(1.0)	0(1.0)	0(1.0)	0(1.0)
2, 4, 6-Glc → 3)Glc (1→	1.9(2.1)	2.3(2.0)	2.1(1.7)	2.0(1.9)	2.1(1.8)	2.0(2.1)
2, 4, 6-Gal → 3)Gal(1 →	0.9(1.1)	1.4(1.3)	1.1(1.0)	1.0(1.3)	1.2(1.0)	1.1(1.1)
2, 3, 4-Glc → 6)Glc(1 →	0.9(1.0)	1.11(1.0)	0.8(0.8)	0.9(0.9)	1.0(0.9)	0.9(1.0)
2, 3, 6-Glc → 4)Glc(1 →	2.1(2.3)	2.4(2.2)	2.3(2.1)	2.3(2.4)	2.4(2.3)	2.0(2.1)
2, 3-Glc → 4)Glc(1 →	2.0(1.1)	2.0(0.9)	2.0(0.9)	2.0(1.1)	2.0(1.0)	2.0(1.1)

The numbers in parentheses refer to values for depyruvylated polysaccharides. Glc, glucitol; Gal, galactitol

Methylation analysis of the native polysaccharides gave 5 peaks (Figure 2) corresponding to (a) 2, 4, 6-tri-*O*-methyl-D-glucitol, (b) 2, 4, 6-tri-*O*-methyl-D-galactitol, (c) 2, 3, 4-tri-*O*-methyl-D-glucitol, (d) 2, 3, 6-tri-*O*-methyl-D-glucitol, and (e) 2, 3-di-*O*-methyl-D-glucitol in the ratio of approximately 2:1:1:2:2. The depyruvylated and subsequently methylated polysaccharide showed the appearance of a sixth peak (f) corresponding to 2, 3, 4, 6-tetra-*O*-methyl-D-glucitol. This also resulted in a decrease in the size of the peak for 2, 3-di-*O*-methyl-D-glucitol by half. This gave the information that the pyruvate residue was attached to 2, 3-di-*O*-methyl-D-glucitol moiety by *O*-4 and *O*-6 linkages. The depyruvylated polysaccharides contained sugars (a), (b), (c), (d), (e) and (f) in an approximate ratio of 2:1:1:2:1:1 respectively.

Methylation analysis of the native and depyruvylated polysaccharides of these five *Rhizobium* strains revealed that the polysaccharides contain (1→3), (1→6), (1→4), (1→4, 1→6) linked D-glucose residues, (1→3) linked D-galactose residue and non-reducing terminal D-glucose attached with pyruvate.

Compositional homogeneity in extracellular polysaccharides of different strains of the same species viz. *R. meliloti* has been reported (Amarger *et al.*, 1967). The structural studies in this work revealed that five polysaccharides from two different species of *Rhizobium* viz. *R. trifoli* and *R. meliloti* have similar structures and their structures are similar to that of succinoglycan, a succinic acid-containing water-soluble, extracellular polysaccharide elaborated by *Alcaligenes faecalis* var. *myxogenes* 10C3. Detailed compositional and structural studies on succinoglycan have been carried out by Misaki *et al.* (1969), Harada *et al.* (1979) and Hisamatsu *et al.* (1980).

Table 2 compares the types of sugars (and the linkages between them) among the polysaccharides of *Rhizobium* and succinoglycan.

It was interesting to note that *R. trifolii* and *R. meliloti* which form effective nodules on two different hosts viz. clover and alfalfa respectively, had the same polysaccharide structure. The results reported in this paper, thus clearly show that the exopolysaccharides have no involvement in rhizobial host specificity. Interestingly, the polysaccharide structure of 5 different strains of *Rhizobia* is similar to that of succinoglycan produced by *Alcaligenes faecalis* var. *myxogenes* 10C3, an organism belonging to a genus altogether different from *Rhizobium*. Members of the genus *Alcaligenes* neither make nodules on the plant roots nor fix atmospheric nitrogen.

### Acknowledgements

This work was supported financially by UNESCO. The author is very thankful to Prof. T. Harada, Dr. A. Amemura and Mr. M. Hisamatsu of the Institute of Scientific and Industrial Research, Osaka University, Japan, for providing facilities, many suggestions and technical help respectively. Thanks are also due to Prof. Y. Maruyama, University of Tokyo and Prof. M. Yatazawa, University of Nagoya for gifts of *Rhizobium* strains.

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## Growth of *Mycobacterium smegmatis* in minimal and complete media

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MS received 2 September 1980

**Abstract.** The growth patterns of *Mycobacterium smegmatis* SN2 in a minimal medium and in nutrient broth have been compared. The growth was monitored by absorbancy (Klett readings), colony forming units, wet weight and content of DNA, RNA and protein. During the early part of the growth cycle, the bacteria had higher wet weight and macromolecular content in nutrient broth than in minimal media. During the latter half of the growth cycle however, biosynthesis stopped much earlier in nutrient broth and the bacteria had a much lower content of macromolecules than in the minimal medium. In both the media, a general pattern of completing biosynthesis rapidly in the initial phase and a certain amount of cell division at a later time involving the distribution of preformed macromolecules was seen. The possible adaptive significance of this observation has been discussed.

**Keywords.** *Mycobacterium smegmatis*; growth; macromolecular content.

### Introduction

The growth of bacteria under different nutritional conditions has been studied in considerable detail (Doelle, 1969; Nierlich, 1979; Oginski and Umbreit, 1959; Payne and Weibe, 1978; Sokatch, 1969). However, comparatively little is known regarding mycobacterial nutrition and physiology (Barksdale and Kim, 1977; Ramakrishnan *et al.*, 1972; Ratledge, 1976). Our interest in this field was aroused by the observation that the burst size of mycobacteriophage I3 (Gadagkar, 1979) depends on the medium in which the host bacteria are being grown. The phage grows well on *Mycobacterium smegmatis* growing in a complete medium (nutrient broth) but very poorly if the bacteria are growing in a synthetic minimal medium. With the hope of gaining an insight into the reasons for this difference in phage growth, we have compared the growth of the host bacterium *M. smegmatis* in complete (nutrient broth) and that in minimal media. It is evident from the results described in this paper that such a comparison not only suggested a possible clue to the effect of the medium on phage growth but also revealed a number of interesting factors concerning bacterial growth *per se*.

### Materials and methods

#### *Growth Media*

- a) Minimal medium (Younmans and Karlson, 1947). L-asparagine, 5g; potassium dihydrogen phosphate, 5.9g; potassium sulphate, 0.5g; citric acid, 1.5g;

- magnesium carbonate, 0.6g; glycerol, 20ml and distilled water to make 1000 ml; pH was adjusted to 7.2 and the medium was supplemented with Tween-80, (0.2% v/v).
- b) Nutrient broth. peptone, 10g; sodium chloride, 5g; beef extract, 5g; distilled water to make 1000 ml; pH was adjusted to 7.4 and the medium was supplemented with Tween-80, (0.2% v/v).
  - c) Nutrient agar. Nutrient broth with 1.5% agar-agar.

#### *Buffer*

10 mM potassium phosphate buffer with 150 mM NaCl and 1 mM EDTA, pH 7.4 is referred to as the standard buffer in this paper.

#### *Growth curves*

For all experiments described in this paper, *Mycobacterium smegmatis* SN2 was grown in 100 ml of minimal medium or nutrient broth taken in a 250 ml Erlenmeyer flask with a side arm. A 1% inoculum of bacteria, freshly grown upto 24 h in minimal medium was added and incubated in a rotary shaker at 37°C. At 2 h intervals, the absorbance of the culture was monitored in a Klett-Summerson colorimeter (using filter no. 54). At the same time a small portion of the culture was removed and plated on nutrient agar for the determination of colony forming units.

#### *Determination of wet weights and dry weights*

At different time intervals, a sample of the culture was centrifuged at 12000 g for 30 min, washed with 0.5% NaCl and the pellet weighed to get the wet weight. For the determination of dry weights, cells were dried in an oven at 90°C cooled, weighed and the process repeated, until a constant weight was reached.

#### *Determination of protein, DNA, and RNA contents*

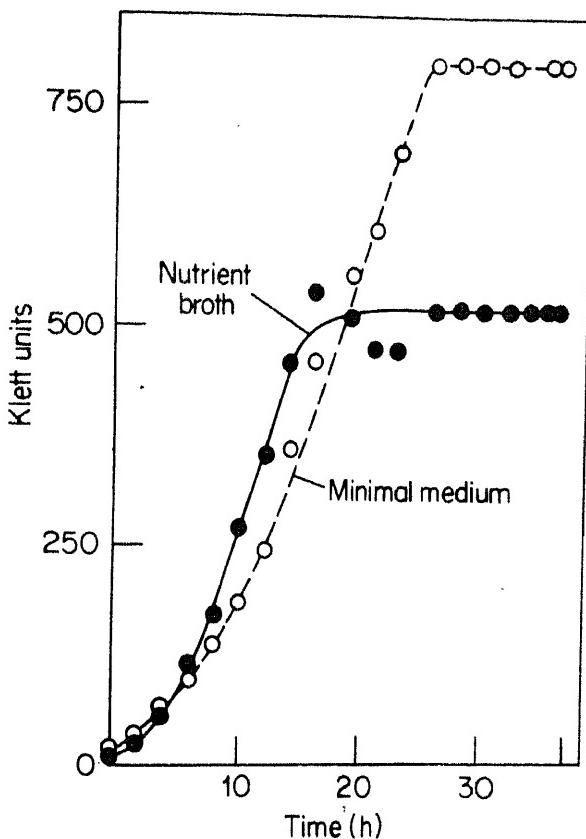
Protein, DNA and RNA contents from packed wet cells were determined according to the method of Schneider (1957). Washed cells were suspended at about 1g/10 ml standard buffer and 2 vol. ice-cold 10% trichloroacetic acid were added. After 30 min at 4°C, the mixture was centrifuged at 12000 g for 20 min. The pellet, containing acid-insoluble macromolecules was washed twice with ethanol, suspended in 10 ml of ethanol-ether (3:1) and heated in a water bath at 60°C for 30 min. This was chilled, centrifuged at 12000 g for 15 min at 4°C and the supernatant discarded. The precipitate was suspended in 1 N KOH and incubated in a water bath at 37°C for 20 h to hydrolyse the RNA. To this hydrolysate, 0.4 ml of 6 N HCl and 2 ml of 5% trichloroacetic acid were added and it was incubated for 30 min at 4°C and then centrifuged at 12000 g for 20 min. The supernatant was used for RNA estimation by the Orcinol procedure (Ceriotti, 1955).

The precipitate was suspended in 10 ml of 5% trichloroacetic acid and heated in a water bath at 90°C with occasional stirring for 20 min. It was then cooled to 4°C, retained at that temperature for 30 min and centrifuged at 12000 g for 20 min. This supernatant was used for DNA estimation by the diphenylamine reaction (Burton, 1956) and the pellet was used for protein estimation (Lowry *et al.*, 1951).

## Results

### Growth curve monitored as Klett units

Figure 1 shows typical growth curves of *M. smegmatis* in minimal medium and in nutrient broth. Several observations can be made from the figure:



**Figure 1.** Growth curve of *M. smegmatis* in minimal medium and nutrient broth monitored as Klett Units.

- 1) There was no significant lag period, as this has been deliberately avoided by using a fresh inoculum and a pre-warmed medium.
- 2) Initial growth rates were higher in nutrient broth. This was expected because nutrient broth would contain ready made precursors for macromolecular synthesis unlike the minimal medium.
- 3) Growth stops at about 16-18 h (at a Klett reading of about 500) in nutrient broth, whereas, in the minimal medium growth stops only at about 25 h (at a Klett reading of 800). The reasons for this are not immediately obvious (see below).

### Growth curve monitored by colony forming units

Figure 2 shows typical growth curves monitored by colony forming units in nutrient broth and in minimal medium. The actual data points are shown and the smooth curves (solid and broken lines) have been fitted using the logistic equation, where, N is the number of bacteria at any given time, K, the maximum population reached and r, the intrinsic growth rate. The intrinsic growth rates

$$\frac{dN}{dt} = rN \left[ \frac{K-N}{K} \right]$$

$$r = \frac{\ln \left( \frac{K-N_0}{N_0} \right) - \ln \left( \frac{K-N}{N} \right)}{t}$$

where,  $N_0$  is the initial number of bacteria and t, the time in h were calculated (Odum, 1973) for pairs of adjacent points. Using the mean intrinsic growth rate, the expected population numbers

$$N = \frac{K}{1 + \exp \left[ \ln \left( \frac{K-N_0}{N_0} \right) - rt \right]} \quad \text{were generated.}$$

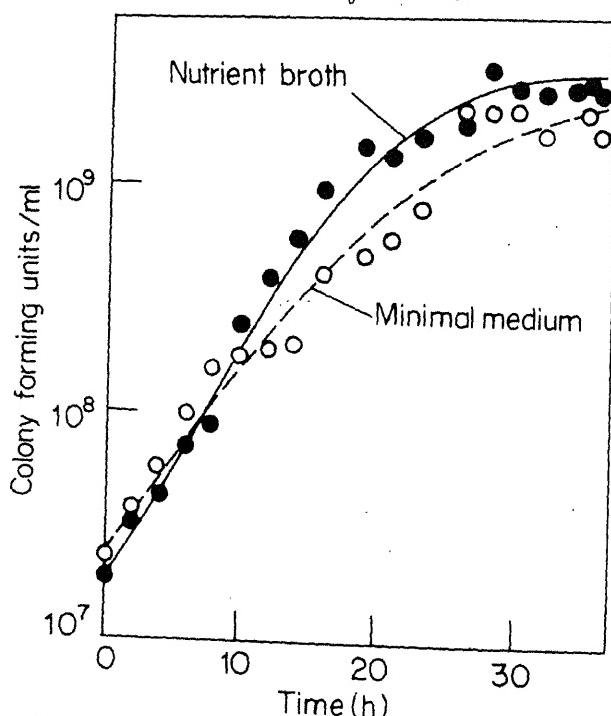


Figure 2. Growth curve of *M. smegmatis* in minimal medium and nutrient broth monitored by colony forming units.

The smooth curves so generated fitted reasonably well to the data points. Besides, mean intrinsic growth rates calculated by the logistic equations agreed well with the realized growth rates at the beginning of the growth curves (table 1) as calculated by the exponential equation

$$N_t = N_0 e^{kt}$$

**Table 1.** Growth rates of *Mycobacterium smegmatis* monitored by colony forming units in minimal medium and nutrient broth.

	Minimal medium	Nutrient broth
Mean intrinsic growth rate	0.19±0.03	0.24±0.03
Mean intrinsic doubling time (h)	3.6 ±0.5	2.9 ±0.3
Realized or specific growth rate at the beginning 0-2 h.	0.19	0.24
Realized or specific doubling time at the beginning 0-2 h.	3.7	2.9

Intrinsic growth rate and doubling time were calculated using the logistic equation while realised or specific growth rate and doubling time were calculated using the exponential equation (see text for details).

In terms of colony forming units the difference between nutrient broth and minimal medium seen in figure 1 was no longer evident. In fact, the cell density was slightly higher in nutrient broth. Thus, there was a discrepancy between Klett readings and colony forming units. A higher Klett reading during the stationary phase in minimal medium inspite of similar numbers of colony forming units (these are confirmed by repeated determinations, table 2) may possibly be due to the secretion of some coloured compound or due to a large excess of dead cells in the minimal medium if they are unable to form colonies. Both these hypotheses have been ruled out because the total cells counted under a phase contrast microscope were not significantly different from colony forming units and the absorbance (Klett units) of the spent medium in either case did not contribute significantly to the Klett readings of the culture (table 2).

**Table 2.** Characteristics of stationary phase cultures of *Mycobacterium smegmatis*

Parameter	Growth medium	
	Minimal medium	Nutrient broth
Absorbance of the culture (Klett units)	828 ± 87 (10) <sup>a</sup>	466 ± 25(10)
Wet wt. (mg/ml)	21.25 ± 2.39 (8)	9.82 ± 0.6(6)
Dry wt. (mg/ml)	3.61 ± 0.51 (8)	1.64 ± 0.2(6)
Water content of cells	83%	83%
Colony forming units/ml	1.5 ± 0.5×10 <sup>9</sup> (9)	3.8 ± 1.7×10 <sup>9</sup> (9)
Total cells/ml <sup>b</sup>	1.2 ± 0.7×10 <sup>9</sup> (10)	3.2 ± 1.6×10 <sup>9</sup> (10)
Absorbance (Klett units) of spent medium	30	3
pH of spent medium	7.2	7.6

<sup>a</sup> The numbers in parenthesis refer to the number of experiments from which the mean and standard deviation have been calculated.

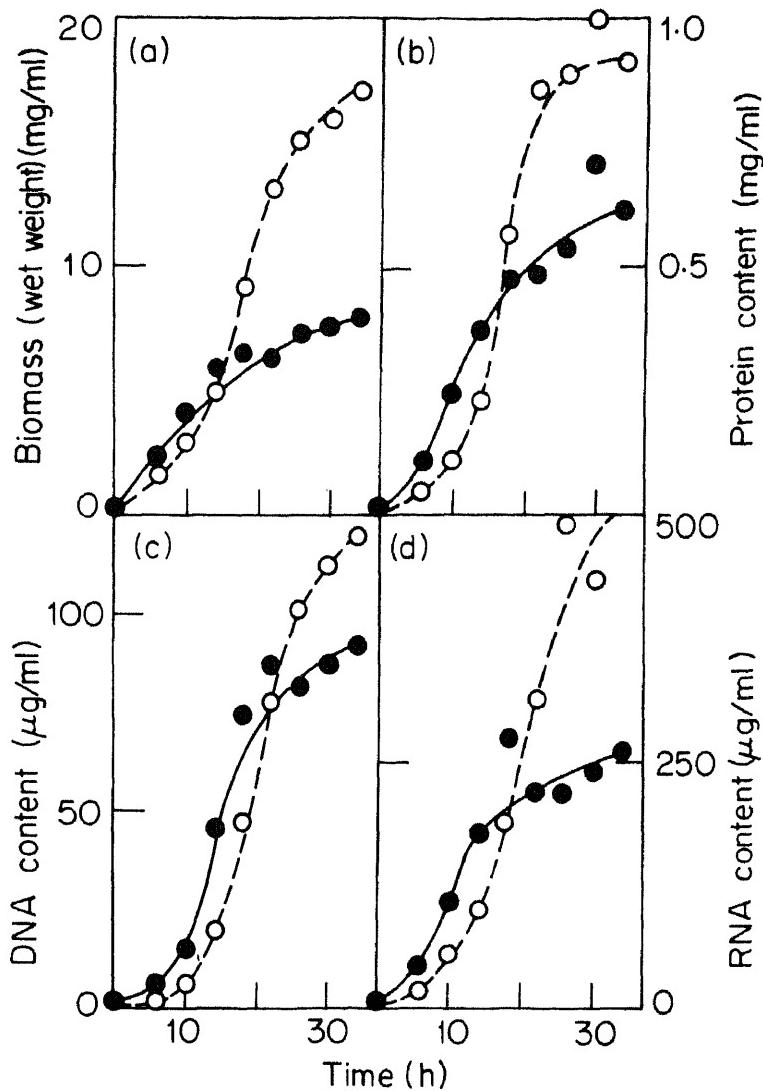
<sup>b</sup> Cell numbers counted in a haemocytometer.

#### Growth curve monitored by wet weight, protein, DNA and RNA contents

Growth curves in minimal medium and nutrient broth monitored by wet weight (mg/ml), protein content (mg/ml), DNA content ( $\mu\text{g}/\text{ml}$ ) and RNA content ( $\mu\text{g}/\text{ml}$ ) are shown in panels A, B, C and D respectively of figure 3. In all the cases growth stopped earlier in time and at a lower density in nutrient broth as compared to that in minimal medium. During the late exponential or early stationary phase, at which time the cultures are generally harvested, the yield in minimal medium was nearly twice that in nutrient broth. Therefore it is better to use minimal medium when large quantities of cells are required, especially since this difference in yield was seen not only in terms of wet weight but also in terms of DNA, RNA and protein contents.

The ratio between colony forming units and Klett readings was reasonably constant in minimal medium. When one Klett reading corresponds to  $1-2 \times 10^6$  cells. However, in nutrient broth, one Klett unit corresponds to about  $1 \times 10^6$  cells in the first half of the growth curve, but more than  $6 \times 10^6$  cells during the latter half (figure 4).

The wet weight and protein, DNA and RNA content per cell in the two media are shown in panels, A, B, C and D respectively of figure 5. As suggested earlier, these were much lower in nutrient broth as compared to minimal medium during the latter half of the growth curve. During the first half of the growth curve however, the situation was the reverse. The cells in nutrient broth had more wet weight, protein, DNA and RNA content than cells in minimal medium (figure 5).



**Figure 3.** Growth curves of *M. smegmatis* in minimal medium and nutrient broth monitored by wet weight, protein, DNA and RNA contents.  
Minimal medium, (O); nutrient broth, (○).

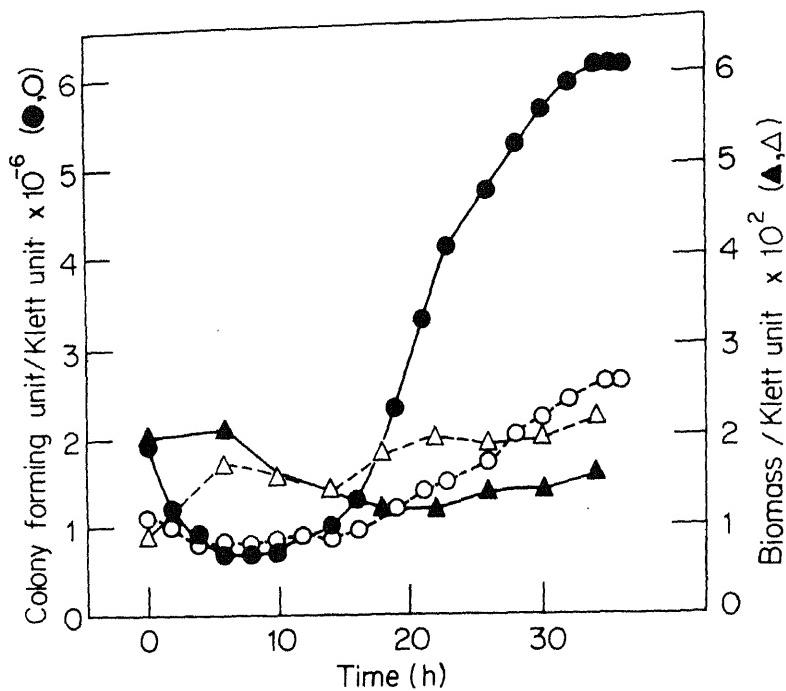
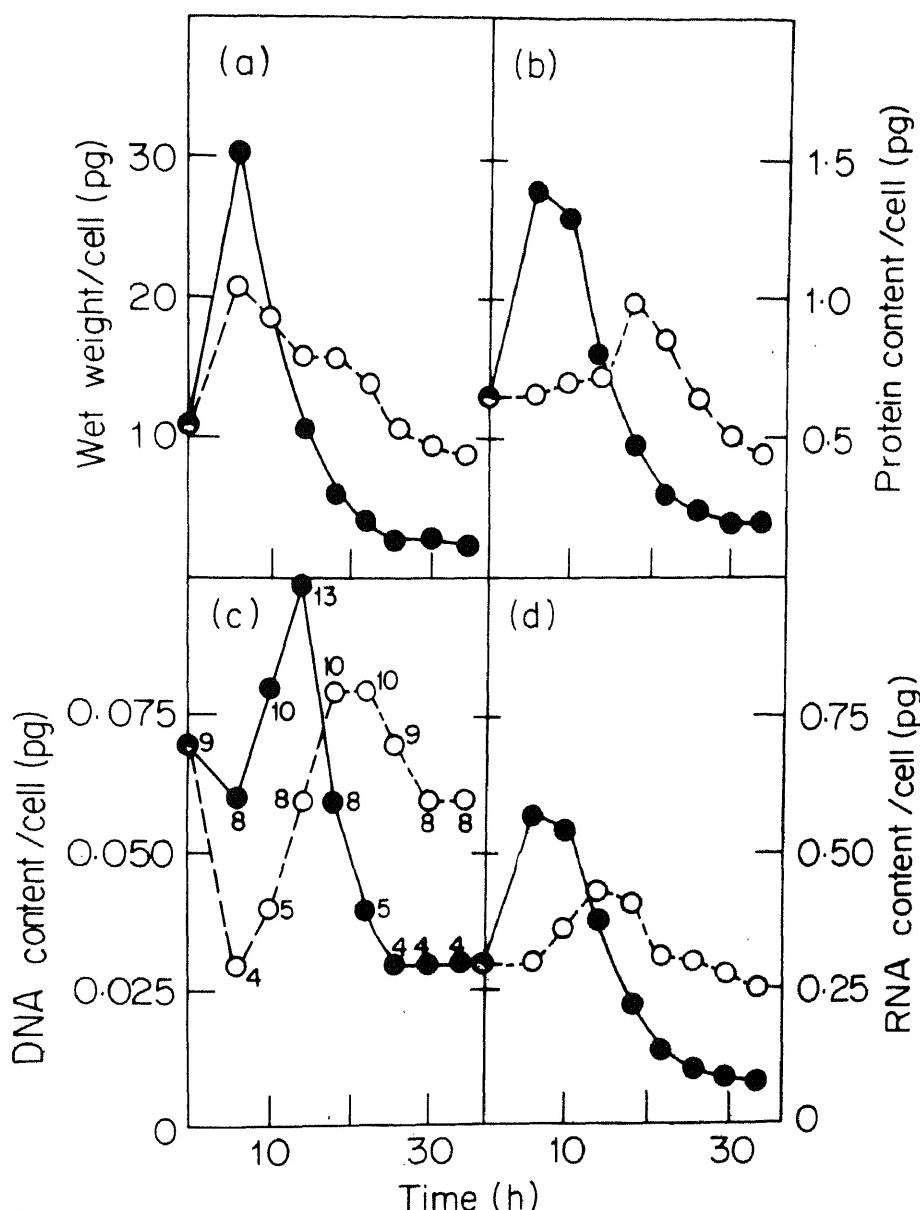


Figure 4. Absorbance (Klett Units) as a function of cell numbers and biomass in cultures of *M. smegmatis* in minimal medium and nutrient broth.

Colour forming unit/Klett Unit —minimal medium, (O); Colour forming unit/Klett unit —nutrient broth, (●); Biomass/Klett unit—minimal broth, (Δ); Biomass/Klett unit—nutrient broth, (▲).

An inspection of figure 5 reveals other interesting features. The fact that the weights and macromolecular contents per cell vary over such a wide range of values suggested that cell division and the biosynthetic machinery were somewhat loosely coupled. In general, two or sometimes three distinct phases can be seen in the life of a cell in a batch culture growth curve (see figure 5):

- (a) an early phase during which synthesis proceeds more rapidly than cell division,
- (b) a later phase where, cell division occurs more rapidly than biosynthesis of cellular components and (c) sometimes, a very early phase that shows constant or decreasing macromolecular content. Such a very early phase is prominent in minimal medium because no preformed growth factors (amino acids, nucleotides etc.) would be present.



**Figure 5.** Mean wet weight (a), protein (b), DNA (c), and RNA (d) contents per cell in cultures of *M. smegmatis* in minimal medium and nutrient broth.

The wet weight, protein, DNA and RNA contents per ml (figure 3) were divided by the numbers of cells per ml (figure 2) to give the mean properties of single cells. In the case of DNA content per cell, the approximate numbers of DNA molecules per cell calculated using Bradley's (1972, 1973) estimate of the molecular weight of *M. smegmatis* DNA ( $4.5 \times 10^9$  daltons) have been shown adjacent to the data points.

Minimal medium, O; Nutrient broth ●.

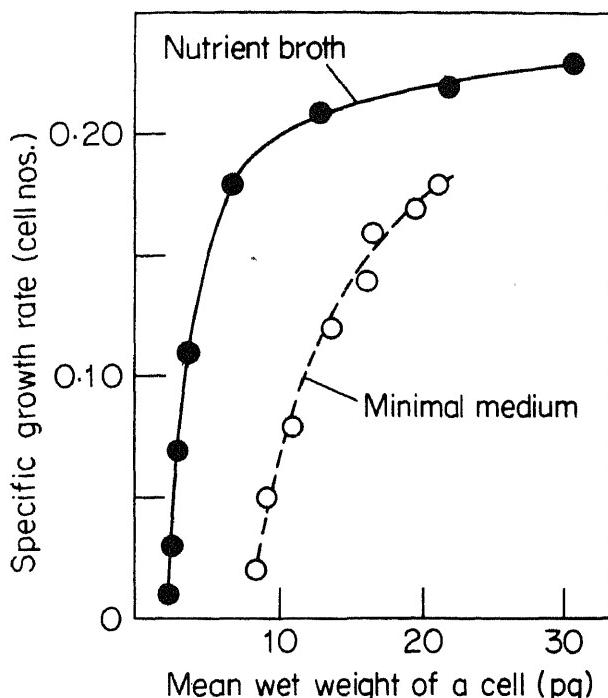


Figure 6. Correlation between mean wet weight per cell and the specific growth rate.

The specific growth rate was calculated by using the data in figure 2 and the equation  $N_t = N_0 e^{kt}$ , where  $N_t$  is the final population,  $N_0$  is the initial population,  $t$ , the time interval and  $k$ , the specific growth rate.

## Discussion

The onset of the stationary phase in bacterial growth curves is generally attributed to the exhaustion of nutrients and/or the accumulation of toxic substances (Stainer *et al.*, 1970). Experiments with *Escherichia coli* have shown that the accumulation of toxic materials is more important in reducing bacterial growth (Landwall and Holme, 1977). Comparison of the yields as dry weight per liter in the two media (table 2) and the nutrient contents in these media shows that only about 10% of the constituents of the media are converted into biomass. Therefore there does not appear to be exhaustion or even a significant depletion of nutrition in either of the media. It is possible that toxic materials would accumulate more rapidly in nutrient broth and hence the early cessation of growth.

The difference between growth in nutrient broth and that in minimal medium as seen by Klett readings may indeed be real and the similarity in the numbers of colony forming units only apparent for the following reasons:

Firstly, growth is the conversion of non-living matter into biomass and therefore cell number taken alone is not likely to be a sufficient index of growth. For example, a cell weighing say,  $X_g$  can divide into two cells of  $0.5 X_g$  each without any growth. Secondly Klett readings are more likely to be a function of biomass rather than cell

numbers for, it is total absorbance that is measured. If these assumptions are valid, it then follows that, a) growth curves monitored by biomass should reflect the pattern of the growth curve as judged by Klett readings rather than colony forming units, (b) the ratio between colony forming units and Klett units should be constant in minimal medium but not in nutrient broth, whereas the ratio between biomass and Klett units should be constant for both the media, and (c) cells in the latter half of the growth curve should be smaller in nutrient broth as compared to those in minimal medium. All these predictions are borne out by the data shown in figures 3-5.

It seems somewhat surprising that even the DNA content per cell shows wide fluctuations, but this may be explained if mycobacterial cells can harbour more than one copy of the chromosome as observed in the case of *E. coli* and *Bacillus subtilis* (Lark, 1966; Watson, 1975). In the latter cases, during the early exponential phase, as many as six copies of the chromosome are known to accumulate which are then distributed to the daughter cells during the late exponential phase. Using the highest estimate of the molecular weight of *M. smegmatis* DNA ( $4.5 \times 10^9$  daltons) reported by Bradley (1972, 1973), the number of chromosome copies can be roughly calculated and is shown with the data points in panel C of the figure 5. If we are justified in using Bradley's estimate of the molecular weight for *M. smegmatis* SN2, then relatively large numbers of chromosome copies seem to accumulate under our experimental conditions. Moreover, the number of chromosome copies (or simply DNA content) does not deplete sufficiently to give only one copy per cell during the late exponential phase. Thus it is possible to start the growth curve with an initial drop in the DNA content per cell. It must be pointed out that since methods employed for the determination of nucleic acids are based on reactions of pentoses, there may be some error due to interference by arabinose from the mycobacterial cell wall. However, the overall pattern of the changes in nucleic acid content is not likely to have been qualitatively altered.

In general, although cell division and the bio-synthetic machinery are coupled, a certain amount of flexibility present in this coupling seems to be exploited to complete biosynthesis as quickly as possible and convert the environmental nutrition into biomass before conditions become unfavourable. As a result, a few cell divisions involving only the distribution of preformed molecules may take place at later times. It may be easier to complete biosynthesis rapidly before cell division because cell division depends on a series of biochemical and morphological changes that would have to take place in a definite sequence. The strategy outlined above is manifest in both the media but much more so in nutrient broth. Here, biosynthesis is completed by 16-18 h but cell division goes on atleast until 28 h giving rise to smaller cells.

The differences in the weight or macromolecular contents per cell between the two media at any given time are not due to lack of separation of the daughter cells. An examination of the cultures under a phase contrast microscope did not reveal any significant numbers of unseparated chains of cells in either medium. It is also unlikely that the above results are due to differences in the rates of cell wall synthesis between the two media. The cells do not weigh consistently more in any one medium; during the early growth phase, cells in nutrient broth weigh more while during the later phase, cells in minimal medium weigh more.

In spite of the apparent loose coupling between biosynthesis and cell division, and inspite of the different phases of growth seen in this system, it is reassuring to note that the general correlation between weight per cell and rate of cell division seen in other systems (Williams, 1971) also holds good in this system (figure 6).

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## The binding of progesterone in different parts of the rabbit uterus during implantation\*

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MS received 12 April 1980; revised 27 June 1980

**Abstract.** Progesterone receptors, both nuclear and cytosolic, were determined in the embryonic and inter-embryonic segments of the rabbit uterus at 6, 7 and 8 day *post-coitum*. At day 6 *post-coitum* a higher concentration of nuclear receptor in the embryonic segment was observed compared with that in the inter-embryonic segment. A reverse situation was observed in the case of cytoplasmic receptors. On the 7th day *post-coitum*, no significant alteration in the concentration of either kind of the receptors was observed. However, on day 8, a higher concentration of both nuclear and cytosolic receptors at the embryonic site was observed compared to that in inter-embryonic segment. Since receptors are influenced only in the immediate vicinity of the blastocyst, it can be suggested that the blastocyst plays a role in the induction of its own implantation. Further, at day 8 increase in receptor concentration at the embryonic site may be related to the presence of decidual tissue at this site.

**Keywords.** Rabbit; uterus; progesterone; receptors.

### Introduction

It is well-known that attachment of the embryo to the maternal uterus is an important aspect of pregnancy. It is also established that hormonal requirements and various morphological and biochemical events occurring in the process of implantation vary considerably among the different mammalian species. In the rabbit, attachment of the embryo to the uterus occurs day 7 *post-coitum* (Enders and Schlappe, 1971) and this process is not dependent on the ovarian oestrogen content (Hafez and Pincus, 1956). However, it has been postulated that oestrogen from the blastocyst is essential for implantation (Dickman *et al.*, 1976; Paychoyos, 1976; Dickman, 1979). The presence of significant amounts of oestrogen and progesterone in the preimplanting blastocyst of rabbit has been established (Seamark and Lutwak-Mann, 1972; Fuchs and Beling, 1974; Dickman *et al.*, 1975) suggesting that these steroids may have a role in the attachment of the embryo to the uterus. Hormonal action is mediated through its binding to receptors in the cytosol and subsequent translocation to the nucleus (Jensen *et al.*, 1968) which can be correlated to subsequent biological changes (Jensen, 1964). The role of these hormones from the blastocyst at the implantation

\* CDRI Communication no. 2741.

site can be delineated by studying hormone receptors in the embryonic and inter-embryonic segments of the uterus during implantation. Variations of estradiol binding in the cytosol at these sites in the rat uterus at 6th day *post-coitum* have been reported (Ward *et al.*, 1978). In a preliminary report that variations of progesterone receptors at day 6 only were examined (Puri and Roy, 1979).

The present report, describes such receptors at the embryonic and inter-embryonic sites of the rabbit uterus prior to, during and after implantation.

## Materials and methods

### Steroids and buffer

[ $1\alpha$ ,  $2\alpha$  -  $^3\text{H}$ ]-progesterone (sp. act, 49 Ci/mmol) was obtained from Radiochemical Centre, Amersham, England. Its purity was checked by thin-layer chromatography on silica gel in benzene: ethyl-acetate (7:3, v/v) system.

In most experiments 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 10% (v/v) glycerol was used.

### Animals

Adult (virgin) female albino rabbits (2.0-2.5 kg) of our Institute colony were used in this study. The animals were divided into three groups consisting of 6 animals in each group. Animals from all the groups were mated with proven bucks and mating was confirmed by the presence of spermatozoa in vaginal smears. Rabbits were killed at day 6, 7 and 8 of pregnancy (day 0=day of mating).

### Preparation of nuclear and cytosol fractions

After sacrifice, uterine horns were excised and kept in ice-cold buffer. The uteri were divided into embryonic and inter-embryonic segments by carefully opening the uterine cornua from the mesometrial side to expose implanting blastocysts. The uterine tissue surrounding the area of each blastocyst was separated as the embryonic site and rest as the inter-embryonic site. Pregnancies with at least three implantation sites were included in the study.

The embryonic and inter-embryonic segments of each group were pooled from 2 animals and weighed separately. Each type of tissue was homogenized with 10 vol. (w/v) buffer in an all glass homogenizer at 0-4°C. The nuclear fraction was obtained by centrifuging the homogenate at 800 g for 10 min. The supernatant was centrifuged at 105,000 g for 60 min to get the cytosol fraction. The nuclear pellet obtained was washed thrice with cold buffer (1.0 ml each time) and then suspended in cold buffer at a concentration of 100 mg tissue/ml. The cytosol was mixed with dextran-coated charcoal suspension (0.05% dextran-70 from Pharmacia and 0.5% Norit A; prepared with 10 mM Tris-HCl, 1 mM EDTA) in the ratio of 1:1 (v/v). The suspension was agitated for 60 min in cold (0-2°C) and centrifuged at 800 g for 10 min. Supernatants thus obtained were immediately used for assay of progesterone binding.

### Measurement of the concentration of progesterone receptor

**Cytosol:** Cytosol receptor assay was done according to the method of Vu Hai and Milgrom (1978a). The aliquots (0.2 ml of cytosol) were mixed with [ $^3\text{H}$ ]-progesterone

(25 nM at the final concentration) solution in Tris EDTA buffer (set A) or [<sup>3</sup>H]-progesterone at the same concentration with 250×progesterone (set B). Unlabelled cortisol at a concentration of 1 µM was used to minimize the interaction of progesterone with corticosteroid binding globulin like protein. Mixtures were incubated for 1 h at 0°C. The free hormone was removed by the addition of 0.3 ml of dextran-coated charcoal suspension, followed by a 10 min incubation at 0°C and centrifugation (800 g for 10 min). Supernatants were decanted into vials and mixed with 12 ml of scintillation cocktail and the radioactivity was measured.

Preliminary experiments demonstrated that the concentration of [<sup>3</sup>H]-progesterone (25 nM) used was sufficient for saturating the cytosol receptors. This equilibrium is reached within 60 min at 0°C (data not shown).

*Nuclei:* The concentration of nuclear progesterone receptor was measured by the method of Vu Hai and Milgrom (1978b). The crude nuclear suspensions (0.2 ml) were incubated for 3 h at 0-4°C with 0.1 ml of [<sup>3</sup>H]-progesterone solution (set A) or [<sup>3</sup>H]-progesterone at the same concentration with 250×progesterone (set A). After subsequent centrifugation, the pellets were washed three times with the homogenizing buffer and extracted twice with 1.0 ml of ethanol each time. The extracts were mixed with 8.0 ml of scintillation cocktail and counted for radioactivity. Preliminary experiments demonstrated that the concentration of [<sup>3</sup>H]-progesterone (25 nM) utilized was saturating and the equilibrium obtained at 3 h of incubation, which was stable for the next 3 h at this temperature (data not shown).

To calculate the specific [<sup>3</sup>H]-progesterone binding by the progesterone receptors radioactivity measured in the supernatants of set B (non-saturable binding) was subtracted from that of the set A (total binding-saturable+non-saturable).

#### *Radioactivity and other measurements*

Radioactivity determination was carried out in a Packard model 3330 Liquid Scintillation Spectrometer; the vials contained 0-0.6 ml of the sample in aqueous phase plus scintillation cocktail (3.25 g PPO; 65 g POPOP, 52 g naphthalene, 300 ml toluene, 300 ml dioxane and 150 ml methanol). The efficiency of the counting was approximately 52%. Quenching corrections were done using the internal standard technique.

DNA content was measured by the diphenylamine method (Burton, 1951) using highly polymerised DNA (Sigma Chemical Co., St. Louis, Missouri, USA) as standard.

#### *Statistical analysis*

The results were subjected to analysis of variance. The difference between the mean value of two groups was calculated by the method of least significant difference. A 'P' value of 0.05 or less was considered to be significant.

### **Results**

It was observed (table 1) that at day 6 of pregnancy the concentration of progesterone nuclear receptors was significantly higher in the embryonic portion compared to that in the inter-embryonic segment of the uterus. At day 7 after coitus no such difference was observed. However, on 8th day *post-coitum*, a significantly higher ( $P<0.02$ ) concentration was obtained at the embryonic site as compared with the inter-embryonic portion.

Table 1. Progesterone nuclear receptors at embryonic and inter-embryonic segments of the rabbit uterus prior to and after implantation. (Results are expressed as fmol/ $\mu$ g DNA).

Days <i>post-coitum</i>	Progesterone bound to the embryonic segment	Progesterone bound to the inter-embryonic segment
6	12.94 $\pm$ 2.87	3.24 $\pm$ 0.77 <sup>a</sup>
7	11.16 $\pm$ 1.77	6.68 $\pm$ 1.70 <sup>b</sup>
8	10.78 $\pm$ 0.59	6.24 $\pm$ 0.85 <sup>c</sup>

Values are mean  $\pm$ S.E. of 3 pooled samples from 2 animals in each pool.

<sup>a</sup>  $P < 0.05$

<sup>b</sup>  $P < 0.02$

<sup>c</sup> Not significant ( $P > 0.05$ ) as compared to embryonic segment.

Table 2 shows that cytosol receptors were significantly higher ( $P < 0.01$ ) at the inter-embryonic segment as compared to the embryonic segment of the uterus. At day 7, after mating no such difference was observed. However, at day 8 *post-coitum*, a significantly higher concentration ( $P < 0.02$ ) at the embryonic segment was observed when compared to the inter-embryonic segment.

Table 2. Progesterone cytosol receptors at the embryonic and inter-embryonic segments of the rabbit uterus prior to and after implantation. (Results are expressed as fmol/ $\mu$ g DNA).

Days <i>post-coitum</i>	Progesterone bound to the embryonic segment	Progesterone bound to the inter-embryonic segment
6	10.10 $\pm$ 0.47	13.33 $\pm$ 0.50 <sup>a</sup>
7	26.71 $\pm$ 3.00	28.18 $\pm$ 0.81 <sup>b</sup>
8	94.23 $\pm$ 10.23	49.12 $\pm$ 2.54 <sup>c</sup>

Values are mean  $\pm$ S.E. of 3 pooled samples from 2 animals in each pool.

<sup>a</sup>  $P < 0.01$

<sup>b</sup>  $P < 0.02$

<sup>c</sup> Not significant as compared to embryonic segment ( $P > 0.05$ ).

## Discussion

The present study demonstrated higher concentration of nuclear receptors at the embryonic site than at the inter-embryonic site of the uterus just before the implantation day (i.e. at day 6 *post-coitum*). The cytosol receptors, however, showed the opposite trend as compared to nuclear receptors. This situation of receptors at the embryonic site of the uterus can be ascribed to the translocation of cytosol progesterone receptors towards the nucleus under the influence of higher concentration of progesterone and estradiol at this site. Progesterone receptors are under dual control of both estrogen and progesterone; estradiol increases cytosolic progesterone receptors, while progesterone promotes its transfer into the nucleus

and also decreases its concentration (Milgrom *et al.*, 1973). Our results suggest that a similar mechanism operates during the changes in the nuclear and cytosolic progesterone receptor content at the implantation site of the uterus caused by progesterone and estradiol. The hormones causing such change at the embryonic site could be produced locally because delivery to the uterus via the general circulation would affect the entire uterus. The obvious local source is the blastocyst. The presence of estrogen and progesterone in the pre-implanting blastocyst has been demonstrated in the rabbit (Singh and Booth, 1978; Angle and Mead, 1979) but their synthesis in the blastocyst has not been shown.

An inflammatory-like reaction (e.g., increase in capillary permeability) has to precede implantation at the prospective site of attachment of the blastocyst (Psychoyos, 1967). Further, it has also been shown that progesterone can act as an antiinflammatory agent. Thus it may be postulated that while progesterone dominance is obligatory for the uterus as a whole it is inhibitory for the local inflammatory-like reaction. Hence, it is necessary to nullify or sufficiently reduce the local progesterone dominance to permit a local inflammatory-like response. Estradiol, which is known to counteract progesterone locally could thus be secreted by the blastocyst.

The above results can be correlated with the hypothesis of Dickman *et al.* (1975, 1976) that mammalian blastocysts secrete steroid hormones, which diffuse out into the adjacent endometrium and induce localized effects in the process of implantation. The verification of diffusion of estrogen from the pre-implanting blastocyst have been indirectly inferred by studying cytosolic estradiol receptors at embryonic site in the rat uterus (Ward *et al.*, 1978). The above possibility has also been confirmed by the increase of various lysosomal (Abraham *et al.*, 1970; Murdoch, 1972; Moulton *et al.*, 1978) and macroscopic reactions (Dickman *et al.*, 1977) at the embryonic site at 6 day *post-coitum* in the rabbit uterus. Irrespective of the origin of steroids it seems likely from these findings that estrogen and progesterone secreted could be the stimulus from the blastocyst to the mother for implantation.

However, as implantation progressed (i.e at day 7 *post-coitum*) there was no significant difference in the concentrations of nuclear and cytosolic receptors at either site. This phenomenon may be correlated with no localized influence or secretion of any of these hormones by the blastocyst at this time and needs further clarification. At day 8 of pregnancy, when trophoblastic invasion of this blastocyst is widely spread, increased levels of both cytoplasmic and nuclear receptors at the embryonic site may directly be attributed to the presence of decidual tissue at this time (De Feo, 1967), which is shown to be influenced by progesterone (Wiest, 1970).

### Acknowledgements

One of the authors (RKP) is thankful to the Council of Scientific and Industrial Research, New Delhi for a Fellowship during the tenure of this work. We are also thankful to Mrs. S. Vaishnavi and Shri R. N. Sarkar for their technical assistance.

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## The cytosol receptors for progesterone in the different parts of rabbit fallopian tube and uterus during ovum transport

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MS received 29 April 1980; revised 14 August 1980

**Abstract.** Progesterone receptors were determined in the cytosol from the ampulla, ampillary-isthmic junction and isthmus of rabbit fallopian tube and uterus of estrus and pregnant rabbits. The receptor levels when compared among its various anatomical segments, were the same in ampulla, isthmis and uterus but maximum in ampillary-isthmic junction. Significant differences were observed in mated animals at 14, 24, 34, 48, 70 and 144 h after coitus. The receptor concentrations in portions of the fallopian tube showed no significant change between 14 and 24 h after coitus, except for a decrease in ampillary-isthmic junction at 24 h. At 34 h the concentration of receptor further decreased in all parts of the tube. At 48 and 70 h after coitus, receptor concentrations decreased gradually in ampulla and ampillary-isthmic junction, while isthmus showed a gradual increase. At 144 h, the receptor concentration showed no further change in ampulla and ampillary-isthmic junction; however, isthmus showed a decline. The uterine receptor concentration declined steadily from estrus till 70 h after coitus, however, it was increased at 144 h. The dissociation constant ( $K_d$ ) of cytosol receptor in all the tissues at estrus and during early pregnancy was found similar. The implications of these changes in relation to the normal ovum transport have been correlated in this paper.

**Keywords.** Progesterone; receptors; fallopian tube; ovum transport; rabbit.

### Introduction

The process of egg transport is an important event in reproduction and is regulated by steroid hormones (Chang and Harper, 1966; Harper 1966; Boling, 1969). The concentration and intracellular distribution of receptors have been reported to vary according to the physiological state of the animals (Martil and Psychoyos, 1976; Lee and Jacobson, 1971; Clark *et al.*, 1972). An estimation of progesterone-receptor levels and a study of their regulation in the fallopian tube and uterus may help in our understanding of the molecular events in the critical period after ovulation.

Earlier studies have shown the presence of progesterone binding macromolecules in rabbit uterus (Rao *et al.*, 1973; Faber *et al.*, 1973; McGuire and Banso, 1972; Davies *et al.*, 1974). The presence of specific receptors for progesterone in human (Fuentealba *et al.*, 1975), chick (Toft and O'Malley, 1972; Sherman *et al.*, 1970) and rabbit oviducts (Muechler *et al.*, 1976) has also been reported. However, El-Banna and Sacher (1977) could not detect cytosolic progesterone receptors in the rabbit and oviduct tissue.

In the present investigation progesterone receptor levels in the cystosol of different segments of rabbit fallopian tube have been studied in relation to ovum transport

and their possible correlation with plasma-estradiol and progesterone levels have been examined.

## Materials and methods

### Animals

Sexually mature (virgin) albino rabbits (2.5 to 3.0 kg) of our Institute colony kept under uniform husbandry conditions at  $26 \pm 2^\circ\text{C}$  were used in this study. The animals were divided into 7 groups; each group consisted of 6 animals. The first group was sacrificed as such while the others were mated with two fertile bucks and the mating was confirmed by the presence of spermatozoa in vaginal smears. Animals of each group were sacrificed at 14, 24, 34, 1 $\frac{1}{2}$ , 70 and 144 h after mating.

### Preparation of cytosol

The tissues were removed, chilled in ice-cold Tris-EDTA buffer (0.01 M Tris-HCl and 0.001 M EDTA, 0.25 M sucrose, pH 7.4). Both oviducts and uteri were excised, trimmed to remove the adhering fat and rinsed in cold Tris-EDTA buffer. Oviducts of all groups were separated into ampulla, ampullary-isthmic junction and isthmus as described elsewhere (Puri and Roy, 1980a). From each group different parts of the oviducts and uteri were weighed separately, minced and homogenized in 10 vol of cold buffer using an all-glass homogenizer at 0-4°C. Homogenates were centrifuged at 800 g for 10 min to get the nuclear pellet which was used for the measurement of DNA (Burton, 1956). The supernatant was centrifuged at 105,000 g for 1 h in an IEC/B-60 (International Equipment Co., Needham Heights, Massachusetts, U.S.A.) ultracentrifuge. The cytosol was mixed with dextran-coated charcoal suspension (0.05% of dextran T-70 from Pharmacia, Uppasala Fine Chemicals, Sweden and 0.5% Norit A, prepared with 0.01 M Tris-HCl, 0.001 M EDTA buffer, pH 7.4) in the ratio of 1:1 (v/v). The suspension was agitated for 60 min in the cold (0-4°C) and centrifuged at 800 g for 10 min. Supernatants thus obtained were immediately used for the assay of progesterone binding.

### Determination of progesterone binding to the cytosol receptor

The receptor assay was carried out according to the method of Vu Hai and Milgrom, (1978) by incubating 25 nM [<sup>3</sup>H]-progesterone (1 $\alpha$ , 2 $\alpha$ , (n)-[<sup>3</sup>H]-progesterone (specific activity 49 Ci/mmol) purchased from the Radiochemical Centre, Amersham, England) at 0-4°C for 60 min (Set A); the non-specific binding was determined by parallel incubation in the presence of a 250-fold excess of cold progesterone (Set B). Unlabelled cortisol at a concentration of 1  $\mu\text{M}$  was used to minimize the interaction of progesterone with corticosteroid binding globulin.

Preliminary experiments demonstrated that the concentration [<sup>3</sup>H]-progesterone (25 nM) used was sufficient for saturating both oviductal and uterine cytosol receptors (data not shown). The optimum incubation time at 0-4°C for [<sup>3</sup>H]-progesterone exchange was also determined in all the segments of the tube and uterus (data not shown). It appeared that cytosols from ampulla and ampullary-isthmic junction segments attained equilibrium after 30 min of incubation while isthmus and uterus did so at 60 min. The maximum specific binding remained stable till 2 h of incubation. Therefore, 1 h was chosen as the optimum time for progesterone cytosol receptor assay at 0-4°C.

At the end of the incubation, progesterone bound to its receptor was separated by incubating with dextran-coated charcoal for 10 min in the cold; then it was centrifuged at 800 g for 10 min. The supernatants were decanted into vials and counted for radioactivity.

#### *Radioactivity measurement*

Radioactivity determinations were carried out in a Packard Model 3330 liquid scintillation spectrometer; the vials contained 0.6 ml of the sample in an aqueous phase plus 12 ml of scintillation fluid (naphthalene 52 g; 2.5 diphenyloxazole, 3.25 g 1,4-bis-[2(5-phenyl-oxazolyl)] benzene, 65 mg; methanol 150 ml; toluene 300 ml and dioxane 300 ml). The efficiency of counting was 52%. Quenching corrections were done using the internal standard technique.

#### *Determination of the dissociation constant*

$K_d$  was determined by incubating cytosols from various tissues with different concentrations of [ $^3$ H]-progesterone (1-10 nM for 1 h at 0.4°C (Set A)). Parallel incubations were also done containing at 250-fold excess of cold progesterone and 1  $\mu$ M of cortisol (Set B). Specific binding was calculated as the difference between bound radiolabelled hormone of these two types of incubations.  $K_d$  was calculated from a Scatchard plot (Scatchard, 1949) of specific progesterone binding. The results were expressed as fmol of bound progesterone per  $\mu$ g DNA and were statistically analysed using the analysis of variance. The difference between the mean values of two groups was calculated by the method of least significant difference ( $P$  value of 0.05 or less was considered to be significant).

#### **Results**

The differences in the binding of [ $^3$ H]-progesterone and the calculated  $K_d$  in different segments of the oviduct and uterus are given in table 1. It appears that at estrus

**Table 1.** Progesterone receptors in the cytosol in the different parts of the fallopian tube and uterus of rabbits in estrus.

Tissue	Number of animals	$K_d \times 10^{-9} M$	fmol bound progesterone per $\mu$ g DNA
<b>Fallopian tube</b>			
i) Ampulla (A)	6	$2.25 \pm 0.09$	$20.95 \pm 2.38$
ii) Ampullary-isthmic junction (AIJ)	6	$2.10 \pm 0.04$ (vs A, $P > 0.05$ )	$33.16 \pm 1.30$ (vs A, $P < 0.01$ )
iii) Isthmus (I)	6	$2.15 \pm 0.09$ (vs A and AIJ $P > 0.5$ )	$23.8 \pm 0.52$ (vs A, $P > 0.05$ ) (vs AIJ, $P < 0.01$ )
Uterus	6	$1.75 \pm 0.26$ (vs A, I and AIJ $P > 0.05$ )	$25.95 \pm 0.85$ (vs A, $P > 0.05$ ) (vs AIJ, $P < 0.02$ ) (vs I, $P > 0.05$ )

Values are mean  $\pm$ S.E.

similar levels of receptors are obtained in ampulla, isthmus and uterus but the concentrations are maximum in ampullary-isthmic junction. The dissociation constants in the uterus and in all the three segments of the tube showed no significant difference.

The pattern of receptor distribution in the above tissues during different post-coital periods are presented in table 2. The ampulla and ampullary-isthmic junction

**Table 2.** Progesterone cytosol receptors in different parts of the fallopian tube and uterus during the transport of ovum.

(Results are expressed as fmol bound progesterone/ $\mu$ g DNA)

Tissue	Post-coitum (h)					
	14	24	34	48	70	144
<b>Fallopian tube</b>						
i) Ampulla	17.39 $\pm$ 2.01	14.34 $\pm$ 3.69	7.53 $\pm$ 0.73	5.72 $\pm$ 0.28	5.47 $\pm$ 0.35	5.92 $\pm$ 0.37
ii) Ampullary- isthmic junction	29.71 $\pm$ 3.24	16.39 $\pm$ 2.38	9.59 $\pm$ 0.54	7.14 $\pm$ 0.56	5.76 $\pm$ 0.53	7.95 $\pm$ 1.61
iii) Isthmus	15.54 $\pm$ 2.47	17.23 $\pm$ 2.35	8.78 $\pm$ 1.55	11.82 $\pm$ 1.58	14.54 $\pm$ 2.60	7.13 $\pm$ 0.25
Uterus	17.20 $\pm$ 1.95	11.76 $\pm$ 0.34	9.25 $\pm$ 2.56	8.62 $\pm$ 2.62	5.51 $\pm$ 0.24	9.97 $\pm$ 1.11

Values are mean  $\pm$ S.E.; number of animals in each group=6.

portions of the tube show no significant change at 14 h after mating; however, the value in isthmus is slightly decreased. At 24 h, although ampulla and isthmus showed no significant alteration over the 14 h group, the ampullary-isthmic junction value is decreased ( $P<0.01$ ). Further at 34 h the values in all the portions significantly declined ( $P<0.01$ ). Thereafter, the receptor concentration in ampulla and ampullary-isthmic junction showed no further change till 70 h after coitus; however isthmus showed a trend to increase. 144 h after mating ampulla and ampullary-isthmic junction showed no significant alteration over the 70 h group but the value in isthmus decreased significantly. The level in the uterine receptor showed a steady decline till 70 h; however, it increased at 144 h over the 70 h group. At different hours after mating the dissociation constants ranged from  $1.75 \pm 0.2 \times 10^{-9}$  M to  $2.61 \pm 0.35 \times 10^{-9}$  M and were not altered significantly in all the tissues studied.

## Discussion

The above findings showed that significant differences occur in the progesterone receptor concentrations in different parts of the fallopian tube and the uterus. These differences cannot be attributed to the changes in the kinetics of hormone binding to the receptor as no significant difference in  $K_d$  was observed. However, the differential sensitivity and retention of this hormone could be a responsible factor.

When progesterone receptors of estrus animals were compared with those of mated animals at different times of mating, significant alterations in different parts of the tube and uterus were noted. All the portions of the tube showed an almost similar pattern of receptor concentration till 144 h after coitus. The  $K_d$  of progesterone receptors in different segments during early pregnancy also showed no significant change, although the values of  $K_d$  as well as receptor concentrations reported here are at variance with those described earlier (Muechler *et al.*, 1977). The discrepancies are primarily, because we used cytosol from which progesterone was removed and different incubation conditions in the exchange assay of receptors.

At 14 and 24 h after mating, when ova are present in ampulla and ampullary-isthmic junction portions of the tube respectively (Gupta *et al.*, 1970; Polidoro *et al.*, 1973), there is no change in receptor concentrations in these portions, corresponding to the unaltered plasma level of progesterone at these times (Spilman and Wilks, 1976). However, the decrease in receptors in ampullary-isthmic junction at 24 h does not correspond to the plasma level. The possibility of involvement of the tissue progesterone level in this decrease cannot be overlooked (Vu Hai *et al.*, 1978). After several hours of blockage of the egg at ampullary-isthmic junction they begin to move into the isthmus between 24 and 36 h after coitus (Polidoro *et al.*, 1973). Therefore, at 34 h when the movement of eggs from ampullary-isthmic junction into the isthmus occurs (Spilman and Harper, 1975; Pauerstein *et al.*, 1974) the sudden fall in receptor concentration in all the parts as obtained here could be correlated with its necessity to release the tubal lock at ampullary-isthmic junction for the transport of the egg. Further, the low level of receptors as compared with control in all the portions of the tube and uterus between 34 and 144 h after mating coincided with increased plasma progesterone levels (Spilman and Wilks, 1976) and the gradual movement of the egg through the isthmus into the uterus (Spilman and Harper, 1975).

On the basis of the above findings and their correlation with plasma progesterone concentration (Spilman and Wilks, 1976), it is concluded that, as plasma progesterone increases, the receptor concentration in tissues decreases. This decrease in cytosol receptors with increasing plasma progesterone could be correlated in either of two ways: (i) cytosol receptors are translocated towards the nucleus; (ii) inactivation of receptor is occurring under the influence of increased progesterone level. Nuclear translocation of the receptor does not fully explain here the disappearance of receptors from the cytosol, as nuclear receptors do not increase at these times (Puri and Roy, 1980b). Thus, the total number of receptors in the above tissues decreases as the plasma progesterone level increases. This inactivation of cytosol receptors has also been described earlier (Milgrom *et al.*, 1972, 1973; Freifeld *et al.*, 1974; Luu Thi *et al.*, 1975; Vu Hai *et al.*, 1977). However, the mechanism of inactivation of progesterone receptors by progesterone is as yet not clear. Our findings further suggest that altered levels of progesterone receptors in different segments of the oviduct and uterus during ovum transport may regulate its action between the time of ovulation and implantation. Furthermore, no definite correlation of plasma estradiol level with the receptors can be offered, as the hormonal level remained unaltered in the post-ovulatory period (Spilman and Wilks, 1976).

The present studies do not support the findings of El-Banna and Sacher (1977) that either progesterone receptors are absent in the tube or that they are already saturated

with endogenous progesterone, which do not exchange with added [<sup>3</sup>H]-progesterone during the incubation; these authors, it appears, used too low concentrations of the hormone and further they did not follow the removal of endogenous hormone by incubation with dextran-coated charcoal before the assaying of receptors. However, our results clearly indicate the presence of progesterone receptors in the fallopian tube.

### Acknowledgement

One of the authors (R.K.P.) is thankful to the Council of Scientific and Industrial Research, New Delhi for providing a fellowship during the tenure of this work and also to Dr Anand O. Prakash for his help in statistical analyses.

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## **Abnormalities in the fine structure of the spermatids of rats injected with cadmium**

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MS received 14 December 1978; revised 12 September 1980

**Abstract.** The degenerative changes in the spermatids as measured by changes in fine structure abnormalities increased with time following injection of Cd<sup>2+</sup> into rat testis. The spermatids in the twelve hours group appear as peculiarly club shaped and elongated structures with one or two small but perceptible vacuoles. The subacrosomal area and the space between the nucleus and the middle piece are seen abnormally dilated. In the 30 day group, the central filaments are the most susceptible unit of 9+2 axoneme complex. The plasma membrane, the cytoplasmic matrix, the mitochondria of the middle piece and the fibrous sheath appear shrunken, discontinuous and degenerative.

**Keywords.** Spermatids; fine structure; rats; Cd<sup>2+</sup> injection; testis.

### **Introduction**

The fine structure of the testis of rat after a single injection of Cd<sup>2+</sup> has been described by Chiquone (1964). He drew attention to the site of action of Cd<sup>2+</sup> and pointed out that the testicular necrosis is due to the effects of this metal upon the endothelium of the vascular bed. Dutt *et al.* (1979) have reported ultrastructural changes in the interstitial cells of Leydig after a single injection of Cd<sup>2+</sup>. The present study provides a description of spermatids exhibiting various abnormalities and degenerative changes in the rat testis as a result of Cd<sup>2+</sup> treatment.

### **Materials and methods**

Adult male rats of Wistar strain (300 g fed stock diet *ad lib*) were maintained on a 12 h:12 h, light:dark schedule. Cd<sup>2+</sup> (0.04 M) was dissolved in distilled water. (Cd<sup>2+</sup> (9.1 mg/kg body weight) was injected subcutaneously as recommended by Parizek (1957). The animals were sacrificed by decapitation at intervals of 6, 12, 48 h and 30 days. Distilled water injected rats served as controls. Small pieces of testis of experimental as well as the control rats were fixed, stained with uranyl acetate followed by lead citrate and examined with JEM-7 A, JEM-110v and JEM-100C electron microscopes (Dutt *et al.*, 1979).

## Results

In the following description, the spermatids, which were identified in the testis of Cd<sup>2+</sup>-injected rats are grouped into the following stages: a) early spermatids and b) late spermatids. Only these stages were used for comparison with the controls. In some cases the cytotoxic damage was so extensive that it was difficult to assign the spermatids to any particular stage. For these reasons abnormalities were described mainly with reference to the acrosomal system, the nucleus, the mitochondria of the middle piece and the axoneme complex of the tail.

### *Early and late spermatids of the controls*

These cells are situated in the area between the primary spermatocytes and the lumen. Their nucleus is spherical and contains uniformly distributed chromatin. The periphery of the nucleus is conspicuous by the presence of a distinct electron dense acrosomal granule enclosed in a vesicle (figure 1). The nucleus of the late spermatid is elongated and flattened, and the mitochondrial sheath surrounding the middle piece is distinct (figures 2 and 3). Various parts of the head such as the outer plasma membrane, the outer and the inner acrosomal membranes and a well developed nucleus with its condensed chromatin are clearly seen. The axial filament complex consists of nine pairs of circumferentially arranged peripheral filaments and a single pair of centrally placed tubular filaments. Some electron dense material is always seen between the peripheral and the central filaments. The flagella contain additional structures at different levels. Thus, the spermatids possess mitochondria, coarse fibres surrounding the middle piece and a dense fibrous sheath (figure 4). The axial filaments are surrounded by a distinct plasma membrane all along their length.

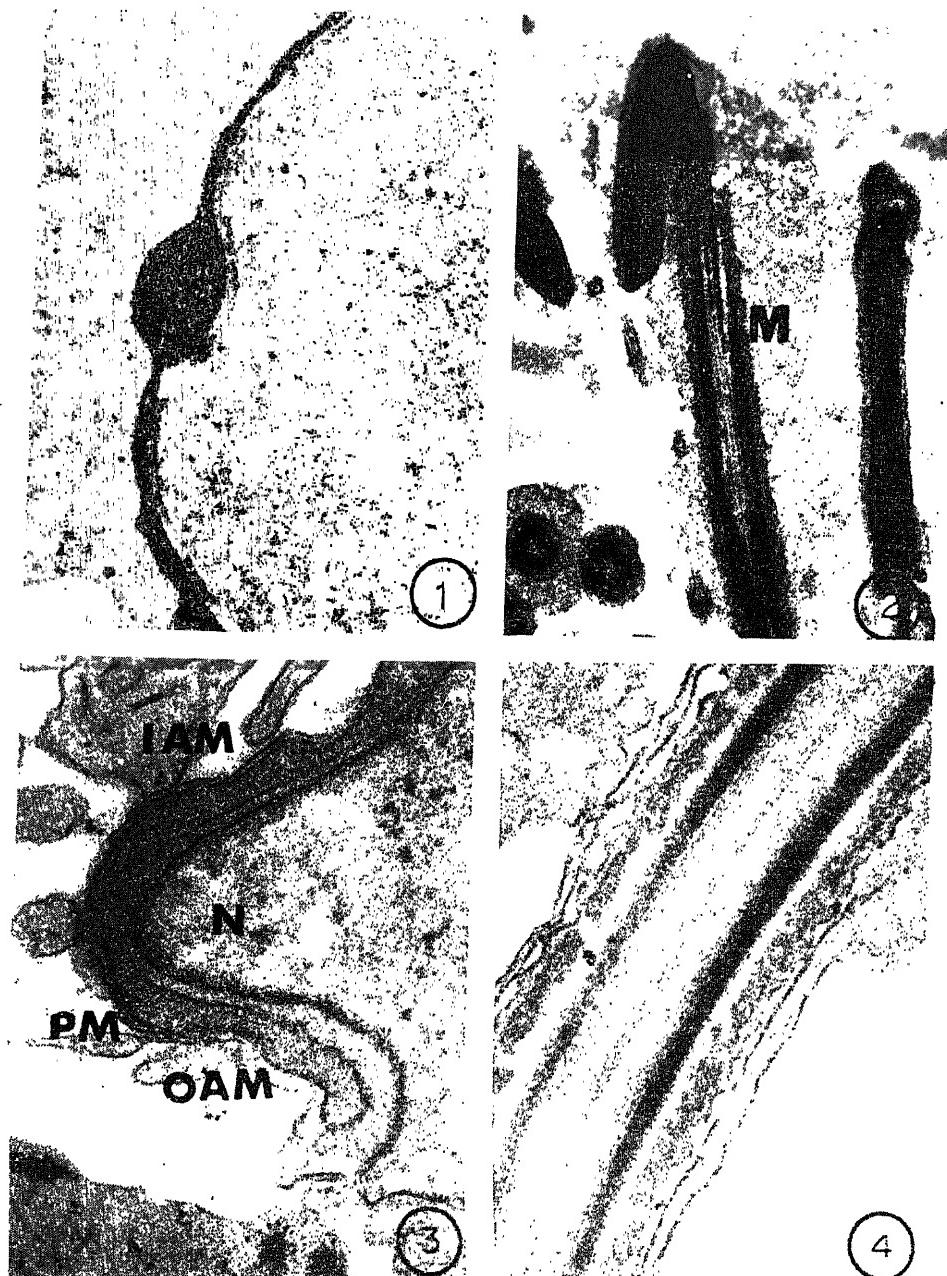
### *Six hour group*

A rather common abnormality of this group is the shrinkage of the nucleus of early spermatids into a flattened structure with a decrease in the stainability of the hemispherical acrosomal granule and the transformation of chromatin into loosely scattered masses within the nucleus (figure 5). Often a number of darkly stained bodies, probably the degenerating nuclei of spermatids, are also observed. They appear to undergo fragmentation into small bodies representing a sign of further disintegration.

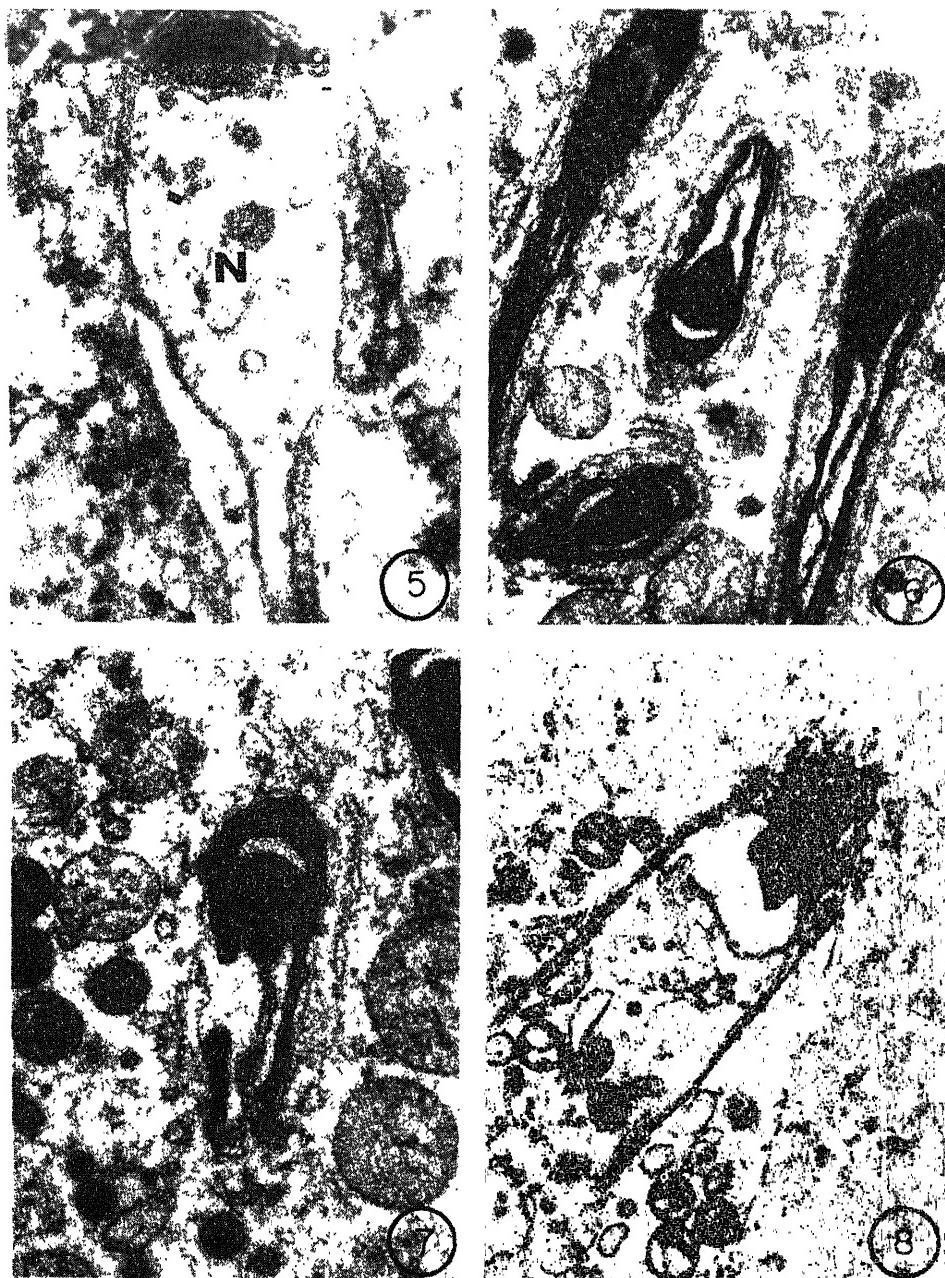
Acrosomal deformation as evidenced by an abnormal growth is observed in certain late spermatids. The acrosome of these cells is seen thick and hypertrophied with thin regions at some points. The oval nucleus undergoes a reduction in size and its posterior region is differentiated into a thread like structure. A perforatorium is absent in many spermatids and where it persists it looks almost empty. No changes are encountered in the sperm tail. The cytoplasmic matrix shows uneven texture. The mitochondria are often blown.

### *Twelve hour group*

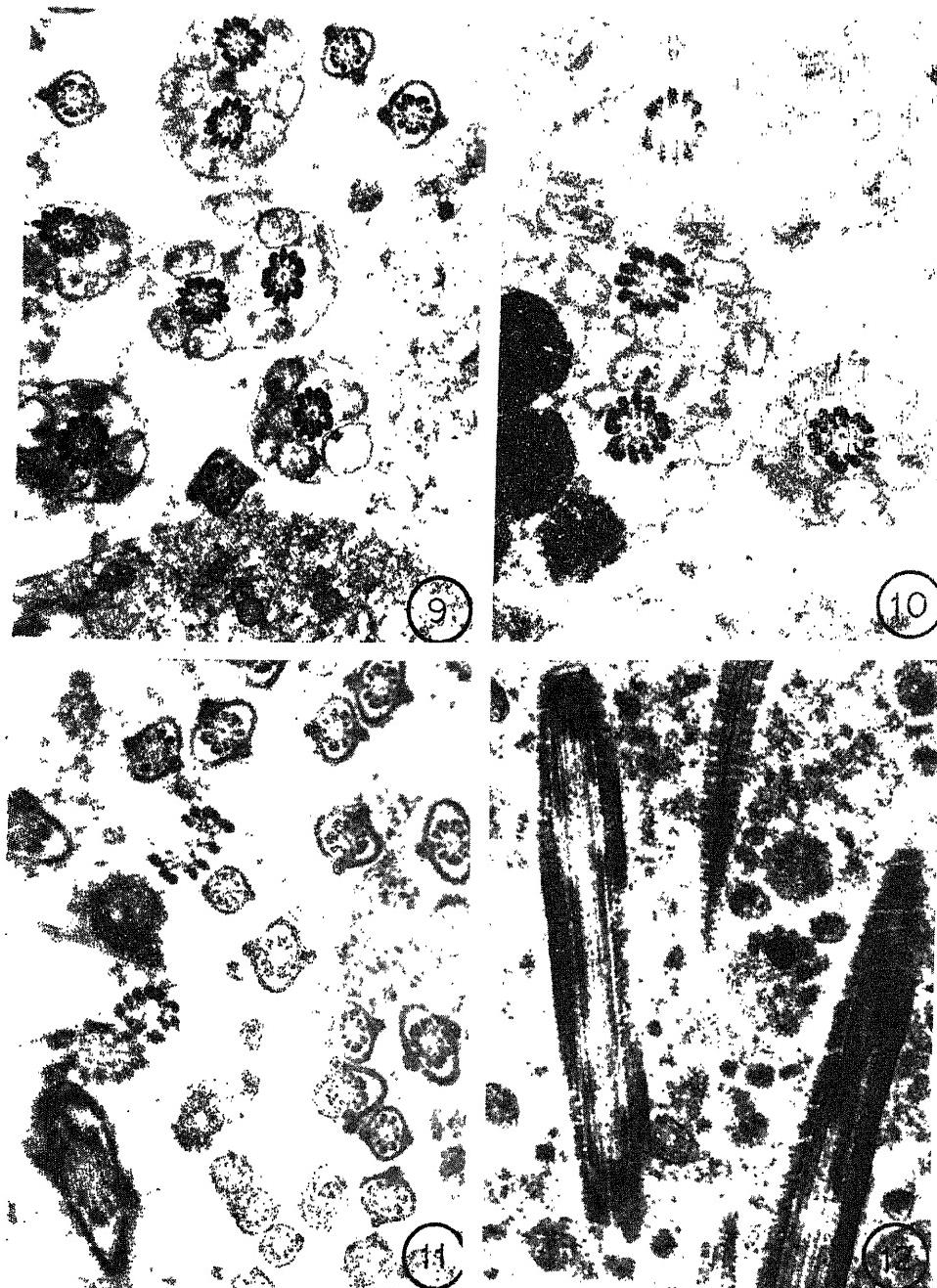
The late spermatids are very much elongated. The acrosomal system appears wavy and is seen parallel to the thread like structure of the nucleus (figure 6). The spermatids appear peculiarly club shaped. The perforatorium is completely lost. A prominent gap is usually noticed in the acrosome (figure 7). The nucleus contains one or two breaks near its flat base. The apical region of the nucleus assumes a conical shape. A few spermatids show a considerably large space beneath their nuclei (figure 8). The spermatid flagellum retains, however, its normal structure.



**Figure 1-4.** 1. A portion of spermatid from the testis of a control rat showing uniformly distributed chromatin and dense acrosomal granule  $\times 16,000$ . 2. Late-spermatid from the testis of a control rat with its intact head, middle piece, mitochondrial sheath and axial filaments (M-mitochondrial sheath)  $\times 8,400$ . 3. Enlarged view of an intact spermatid head to show the plasma membrane, acrosomal membranes and the nucleus (PM-plasma membrane, OAM-outer acrosomal membrane, IAM-inner acrosomal membrane, N-nucleus)  $\times 20,000$ . 4. A longitudinal section through the principal piece of the spermatid tail from a control rat with intact fibrous sheath and central filaments.  $\times 20,000$ .



**Figures 5-8.** 5. A shrunken spermatid head from the testis of rat 6 h after  $Cd^{2+}$  injection showing loosely scattered chromatin masses in the nucleus N-nucleus, Ag-acrosomal granule.  $\times 16,000$ . 6. 12 h after  $Cd^{2+}$  injection. Notice the wavy and dense acrosomal margin of an abnormally elongated spermatid.  $\times 13,000$ . 7. Group same as above showing a prominent gap in the dense acrosomal system around the degenerating nucleus.  $\times 8,900$ . 8. Spermatid showing a large space below its nucleus.  $\times 13,000$ .



**Figures 9-12.** 9. Cross section of the middle piece of the flagellum 48 h after Cd<sup>2+</sup> injection showing loss of cristae in the mitochondria.  $\times 13,400$ . 10. Group same as above. Illustrating the empty mitochondria in the spermatid of the same group as above. 11. 30 days after Cd<sup>2+</sup> injection to show loss of central filaments in the axoneme.  $\times 18,900$ . 12. Spermatid tail revealing discontinuity in its outer fibres.  $\times 18,900$ .

*Forty eight hour group*

The deformative changes in the acrosomal system and in the nucleus are much more pronounced than those in the 12 h group. The longitudinal row of mitochondria along the outer dense fibres shows degenerative changes like reduction in the stainability and complete loss of cristae (figure 9). The plasma membrane surrounding the middle piece is also greatly disrupted and the mitochondria appear empty (figure 10).

*Thirty day group*

The spermatid heads are not commonly observed in this group. When seen they show profiles similar to those of the 12 h group in the process of degeneration. The central filaments of the axoneme complex were not seen in most of the spermatid rats (figure 11). The fibrous sheath was often discontinuous (figure 12), and appears to decrease in size and in staining capacity. There are other signs of disruption including shrinkage and dissolution of the various components of axoneme complex.

**Discussion**

Recently Chowdhury and Steinberger (1975) have shown that the administration of testosterone propionate, dehydrotestosterone or  $5\alpha$ -androstenediol to the rat results in the formation of spermatids upto the stage of spermiogenesis. In the testis of  $Cd^{2+}$  treated rat, necrosis of interstitial cells was reported earlier by Dutt *et al.*, (1978, 1979). These authors have also reported a marked decrease in the level of serum androgens in the  $Cd^{2+}$  treated rats. In view of the dynamic role of androgens in the initiation and maintenance of spermatids, it is believed that the degenerative changes observed in the present studies in the spermatids may be due to deprivation of androgen under the influence of  $Cd^{2+}$ . Whether this effect is due to direct action of  $Cd^{2+}$  on the interstitial cells and if so the question of such mechanism is not clear.

In the testis of  $Cd^{2+}$  injected rats abnormality usually manifests itself in the uncontrolled growth of the acrosome and reduction in the nuclear material. The contact between the nucleus and the middle piece is also destroyed. These changes result in the development of an unusually large space beneath the nucleus. Pleon (1973) also believed that during experimental cryptorchidism the formation of subacrosomal space is the result of a decreased firmness of contact between the acrosome and the nucleus.

Many workers have interpreted the abnormalities of axoneme complex as factors or signal marks contributing to defective motility, thus leading to infertility of sperms due to experimental cryptorchidism (Nagano, 1963), antispermatic agent, Win 18446 (Reddy and Svaboda, 1967), neutron irradiations (Hunt and Johnson, 1971) and sterility in the natural populations (Cooper and Hamilton, 1977). The present work has shown that the flagellum of the sperm of rat is resistant to the influence of  $Cd^{2+}$  upto 12 h after the injection. At 48 h, however, the mitochondria of the middle piece appear as empty membrane bound spaces without cristae or any stainable material in them. The plasma membrane surrounding the fibres is also not seen. In the 30 day group the fibrous sheath reveals discontinuity in structure and loss of uniformity in staining. According to Fawcett (1975) the mitochondria provide the necessary energy for the maintenance of the function of the outer fibres. In view of this it may be interpreted that the mitochondrial degenerative changes probably play

an important role in the process of necrosis of the outer fibres in the Cd<sup>2+</sup> treated rat. Further, in the 30 day group, the central filaments of the tails frequently disappear from view. Such an obscurity of central filaments has been described earlier in the experimentally induced cryptorchid rat (Nagano, 1963). It seems likely, as in the cryptorchid rat, the central filaments are far more sensitive to Cd<sup>2+</sup> than the other components of the axoneme-complex in rat. It is further noteworthy that the longer the duration after Cd<sup>2+</sup> injection the greater is the impairment of axial filaments.

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## Evolutionary trends in the hemoglobins of murine animals

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MS received 18 September 1979; revised 27 October 1980

**Abstract.** The evolutionary origin of murine line based on a phylogenetic tree made on sequence data of  $\alpha$ - and  $\beta$ -hemoglobin chains, followed by the diversity spectrum of hemoglobin genes in two wild species of murine rodents: *Rattus rattus rufescens* (house rat) and *Bandicota indica* (bandicoot rat) has been reported. Each house rat contains six hemoglobin types involving two  $\alpha$ - and three  $\beta$ -chains, which suggests a probable gene duplication at the  $\alpha$ -chain locus and a gene triplication at the  $\beta$ -chain locus. Each bandicoot rat contains one  $\alpha$ - and two  $\beta$ -chains suggesting a probable gene duplication at the  $\beta$ -chain locus. Peptide pattern analysis of the polypeptide chains of these murine hemoglobins further indicates that intraspecies differences among duplicated chains of the same kind are less than interspecies differences among corresponding  $\alpha$ - and  $\beta$ -chains.

**Keywords.** Murine rodents; phylogenetic relationship; hemoglobin gene diversity; gene reiteration.

### Introduction

Rodents presumably arose from the insectivore placental stem (Romer, 1966). From this base, primitive rodents have evolved along many lines of adaptive radiation giving rise to the rat-mouse stock, the most successful of all living mammals. The hemoglobins of these murine animals likewise show a spectrum of wide diversity. For instance, the laboratory mouse and rat show multiple hemoglobin forms which contain several  $\alpha$ - and non- $\alpha$  -polypeptide chains (Ranney and Glueckshon-Waelsch, 1955, Gluecksohn-Waelsch *et al.*, 1957; Russel and Gerald, 1958; Popp, 1969; Travnicek *et al.*, 1971; Gilman, 1972; Garrick *et al.*, 1975). Clearly, the rat-mouse stock is an excellent experimental source for studying the evolutionary trends of hemoglobins. However, any attempt that tries to study the extent of diversity of the hemoglobin genes within any mammalian species, must take into consideration the evolutionary relationships of the hemoglobin genes in that species with those in other mammalian groups. This is necessary since hemoglobin gene diversities appear not only at the level of the species but also at that of broader categories.

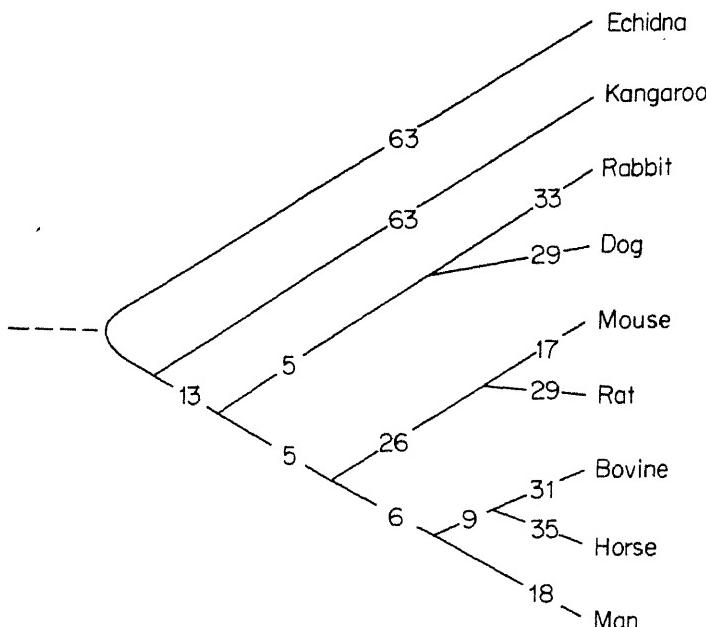
In the present report, we have first located the origin of the murine line of descent by constructing a phylogenetic tree based on the combined sequences of  $\alpha$  and  $\beta$ -chains of the hemoglobins of representative animals species belonging to different mammalian orders. Next, we have structurally characterised the multiple hemoglobin forms of two wild species of murine animals, namely *Rattus rattus rufescens* (house rat) and *Bandicota indica* (bandicoot). Finally, we have made a comparative assessment of the hemoglobin gene diversity in *Rodentia* and those in other mammalian orders.

## Materials and methods

The phylogenetic tree for the combined  $\alpha$ -and  $\beta$ -chain sequences of hemoglobins was constructed by using parsimony methods (Barnabas *et al.*, 1972; Moore *et al.*, 1973; Barnabas *et al.*, 1978). The two murine species under study were trapped in and around Ahmednagar ( $19^{\circ}5'N$ ;  $74^{\circ}45'E$ ). Blood samples of individual animals were collected by cardiac puncture using EDTA or sodium citrate as anticoagulants. Hemoglobin typing was carried out by starch gel electrophoresis using Tris-EDTA-boric acid buffer, pH 8.1, as the gel buffer and boric acid-NaOH buffer, pH 9.0, as the electrode vessel buffer (Huisman, 1963). Hemoglobin variants were isolated by means of both carboxymethyl (CM)-cellulose chromatography (Huisman *et al.*, 1958) and diethylaminoethyl (DEAE)-Sephadex A-50 chromatography (Huisman and Dozy, 1965). Globins were prepared according to the method of Anson and Mirsky (1930). The separation of polypeptide chains was achieved by using the urea-polyacrylamide gel electrophoretic method of Moss and Ingram (1968). Polypeptide chains were isolated following the method of Clegg *et al.*, (1966) with necessary modifications as reported earlier (Pratap *et al.*, 1978). The polypeptide chains were identified as  $\alpha$ -or  $\beta$ -chains by locating the respective characteristic peptides on fingerprints. The globins were aminoethylated prior to mapping the peptides (Jones, 1964). Using the fingerprint of  $\alpha$ -and  $\beta$ -chains of mouse hemoglobin as standard, the tryptophan containing  $\alpha$ -Tp-3 and methionine containing  $\alpha$ -Tp-5 peptides were identified on the fingerprints by appropriate colour tests. The tryptophan containing  $\beta$ -Tp-2 and  $\beta$ -Tp-4 peptides were also similarly identified.

## Results and discussion

In recent years, the idea that sequences of proteins could be used to decipher phylogenetic relationships in animal species has gained fairly good acceptance. Hemoglobin, the principal protein of vertebrate blood has in particular been extensively studied in this context. Consequently, the sequences of  $\alpha$  and non- $\alpha$ -chains of vertebrate hemoglobins have not only been correlated with genetics (Ingram, 1957; Baglioni, 1963) but their variation in different species have been used to derive gene species phylogeny as well (Fitch and Margoliash, 1967; Barnabas *et al.*, 1971; Goodman *et al.*, 1972; Goodman *et al.*, 1974). We have constructed a phylogenetic tree for the hemoglobins of representative animal species of six eutherian orders along with those of a protherian and metatherian (figure 1). From this figure, it is evident that the hemoglobins of eutherian mammals separate after the divergence of those of echidna (Protherian) and kangaroo (Metatherian). In the eutherian line of descent, the hemoglobins of rodent (*Mus musculus* and *Rattus norvegicus*) separate prior to those of ungulates (*Equus caballus* and *Bos taurus*) and a primate (*Homo sapiens*), but after the divergence of those of a lagomorph (*Oryctolagus cuniculus*) and a carnivore (*Canis familiaris*). This is consistent with the presumed origin of rodents (Romer, 1966). It is noteworthy that rat and mouse which belong to the same subfamily *Murinae* show considerable nucleotide replacements (17 for mouse and 29 for rat) in their hemoglobins since the time of their immediate common ancestor (figure 1). This also confirms earlier immunological and DNA hybridisation data (Laird *et al.*, 1959; Sarich, 1972). Furthermore, Morrison and co-workers (1977) suggest that two cricetine genera: *Peromyscus* and *Calomys* stand closer in their hemoglobin make-up to mouse than to rat.

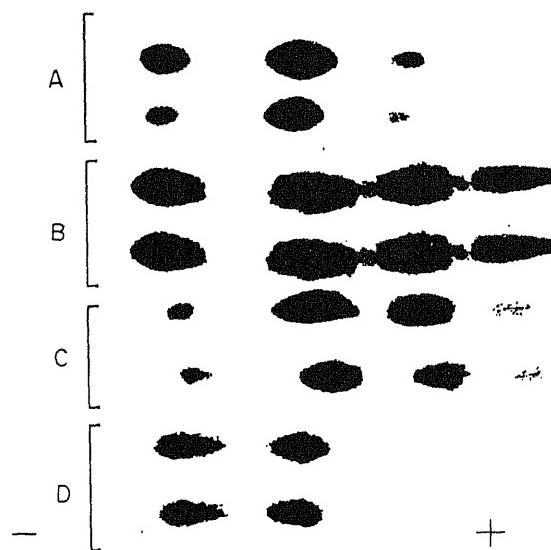


**Figure 1.** An evolutionary tree of combined hemoglobin  $\alpha$ - $\beta$ -chain sequences. Man (*Homo sapiens*) (Braunitzer *et al.*, 1961), horse (*Equus caballus*) (Smith, 1964; Kilmartin and Clegg, 1967), bovine (*Bos taurus*) (Schroeder *et al.*, 1967), rat (*Rattus norvegicus*) (Chus *et al.*, 1975; Garrick *et al.*, 1978), mouse (*Mus musculus*) (Popp, 1967; Popp, 1972), dog (*Canis familiaris*) (Jones *et al.*, 1971; Dresler *et al.*, 1974), rabbit (*Oryctolagus cuniculus*) (von Ehrenstein, 1966; Best *et al.*, 1969), kangaroos (*Macropus giganteus*) (Beard and Thompson, 1971; Air and Thompson, 1972), and echidna (*Tachyglossus aculeatus*) (Whittaker *et al.*, 1972; Whittaker *et al.*, 1973). Numbers represent nucleotide replacements between ancestor and descendant sequences.

### Hemoglobin gene iteration in house rat and bandicoot

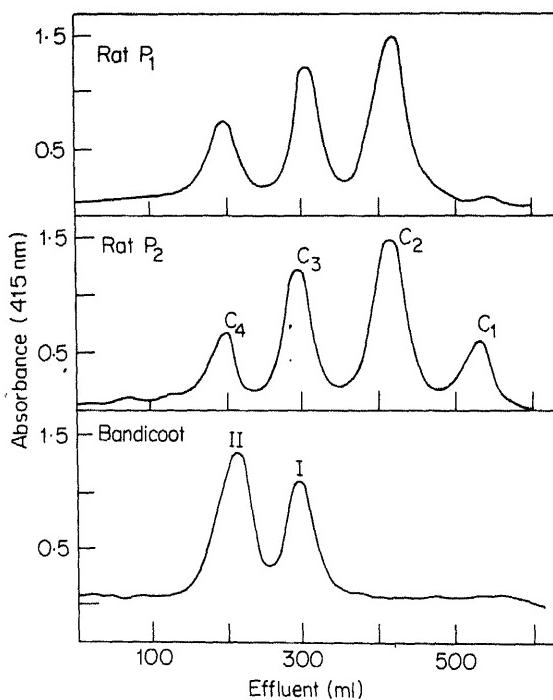
Alkaline starch gel electrophoresis of 1025 blood samples of house rat revealed four hemoglobin components in each referred to as  $C_1$ ,  $C_2$ ,  $C_3$  and  $C_4$  in the decreasing order of their anodic mobilities (figure 2). The relative proportions of each component varied considerably in different samples. For practical purposes, we tentatively characterised phenotypes of the house rat into two broad categories,  $P_1$  and  $P_2$ . In  $P_1$ ,  $C_1$  concentration was very low (2 to 4% of the total) whereas in  $P_2$ ,  $C_1$  concentration varied from 7 to 20%. The bandicoot on the other hand showed 2 hemoglobin components in the ratio 40:60 in each of the 140 samples examined on starch gels.

The hemoglobins from representative samples from each species were subjected to chromatography on CM-cellulose as well as on DEAE-Sephadex A-50 columns. By subjecting each sample to both a cation and an anion exchanger, it was possible to get the component hemoglobins in a pure state. Figure 3 shows the chromatographic profile on DEAE-Sephadex A-50 columns.



**Figure 2.** Starch gel electrophoretic pattern of murine hemoglobins. A. house rat ( $P_1$ ); B. house rat ( $P_2$ ); C. house rat ( $P_2$ ); D. bandicoot

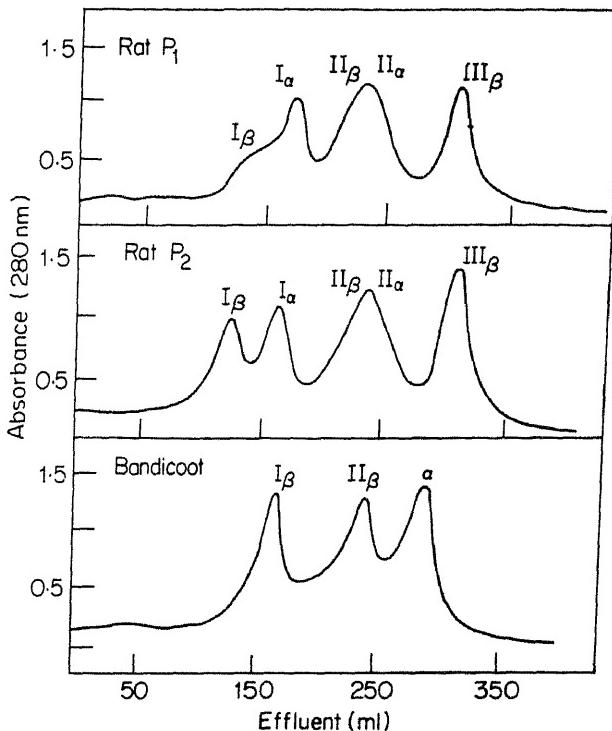
Buffer Gel: Tris-EDTA-Boric acid, pH 8.1 Trough: Boric acid-NaOH, pH 9.0



**Figure 3.** DEAE-Sephadex A-50 chromatographic separation of hemoglobins of house rat ( $P_1$  and  $P_2$ ) and bandicoot. A pH gradient of Tris-HCl buffers (0.05M) was used.

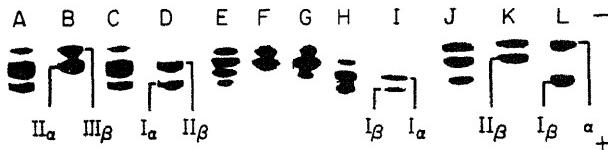
The mode of identification of subunits of component hemoglobins as either  $\alpha$ - or  $\beta$  involved isolation of the polypeptide chains by urea-CM-cellulose chromatography.

The mode of identification of subunits of components hemoglobins as either  $\alpha$  or  $\beta$  involved isolation of the polypeptide chains by urea-CM-cellulose chromatography and subsequent location of typical tryptic peptides in the fingerprints of the aminoethylated globins. Figure 4 presents the urea-CM-cellulose chromatographic profile



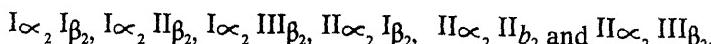
**Figure 4.** Chromatographic separation of polypeptide chains of globins of house rat ( $P_1$  and  $P_2$ ) and bandicoot, by urea-CM-cellulose chromatography.

and figure 5 presents the relative mobilities of different polypeptide chains on urea-polyacrylamide gel electrophoresis. Since the extreme components on chromatographs or electrophoregrams are likely to be least contaminated, we first analyzed these components. Thus  $C_1$  from  $P_2$ ,  $C_2$  from  $P_1$  and  $C_4$  from either  $P_1$  or  $P_2$  from



**Figure 5.** Separation of polypeptide chains of globins in urea-polyacrylamide gel electrophoresis.  
A. house rat ( $P_1$ ) total globin; B. house rat ( $P_1$ )  $C_4$  globin; C. house rat ( $P_1$ )  $C_3$  globin;  
D. house rat ( $P_1$ )  $C_2$  globin; E. house rat ( $P_2$ ) total globin; F. house rat ( $P_2$ )  $C_4$  globin;  
G. house rat ( $P_2$ )  $C_3$  globin; H. house rat ( $P_2$ )  $C_2$  globin; I. house rat ( $P_2$ )  $C_1$  globin;  
J. bandicoot total globin; K. bandicoot Hb-II globin; L. bandicoot Hb-I globin.

house rat hemoglobins were initially analyzed. The C<sub>1</sub> from P<sub>2</sub> on urea-CM-cellulose chromatography separated into two fractions which on fingerprinting could be identified as an  $\alpha$ -chain and a  $\beta$ -chain. These chains were designated as I $\alpha$  and I $\beta$  chains respectively. In naming the polypeptide chains, preceding superscripts have been used to denote more than one copy of the gene according to the nomenclature proposed by Huisman and Schroeder (1970). The C<sub>2</sub> from P<sub>1</sub> on similar treatment showed two major fractions which on structural analysis could be identified at I $\alpha$  and II $\beta$  chains respectively. Similarly C<sub>4</sub> from either P<sub>1</sub> or P<sub>2</sub> separated into two major fractions which could respectively be identified as II $\alpha$  and III $\beta$  chains. The relative mobilities of these polypeptide chains of urea-polyacrylamide gel at alkaline pH are shown in figure 5. Clearly II $\alpha$  and II $\beta$  chains have more or less the same anodic mobility, I $\beta$  having highest and III $\beta$  the lowest. From these observations, it is evident that C<sub>1</sub> represents I $\alpha_2$ , I $\beta_2$ . Similarly C<sub>2</sub> from P<sub>1</sub> has I $\alpha_2$ , II $\beta_2$  as the major component; and C<sub>4</sub> represents II $\alpha_2$ , III $\beta_2$ . Based on the electrophoretic and chromatographic mobilities of different polypeptide chains, we assumed that C<sub>3</sub> from P<sub>2</sub> would contain two molecular form viz I $\alpha_2$ , II $\beta_2$  and II $\alpha_2$ , I $\beta_2$ . Similarly C<sub>2</sub> from either P<sub>1</sub> or P<sub>2</sub> was assumed to contain II $\alpha_2$ , II $\beta_2$  and I $\alpha_2$ , III $\beta_2$ . The structural analysis of these component hemoglobins confirmed our assumption. The components C<sub>2</sub> from P<sub>2</sub> showed four polypeptide chains namely I $\alpha$ , II $\alpha$ , I $\beta$  and II $\beta$ . Similarly C<sub>3</sub> showed four chains namely I $\alpha$ , II $\alpha$ , II $\beta$  and III $\beta$  (figure 5). On the basis of these findings, we believe that there are six molecular forms of hemoglobins in the wild population of *Rattus rattus rufescens*. These molecular forms are:



The two components of the bandicoot were isolated through DEAE-Sephadex A-50 (figure 3) and subjected to urea-CM-cellulose chromatography (figure 4) and urea-polyacrylamide gel electrophoresis (figure 5). The isolated chains were mapped for their tryptic peptides. The two components were found to contain a common  $\alpha$ -chain but differing  $\beta$ -chains designated as I $\beta$  and II $\beta$ . The molecular forms in the bandicoot are  $\alpha_2$ I $\beta_2$  and  $\alpha_2$ II $\beta_2$ .

Mice and rats show both non-allelic and allelic hemoglobin variants. For instance, several alleles at the hemoglobin loci including the ones in which 'breeding unit alleles' contain two DNA sequences are known in the inbred strains of mice (Hilse and Popp, 1968). Starting with the observation that hemoglobins of mice showed a 'single' and a 'diffuse' band (Ranney and Gluecksohn-Waelsch, 1955) on paper electrophoresis, Gluecksohn-Waelsch and co-workers (1957) have subsequently shown that these two traits are controlled by allelic genes. Three hemoglobin alleles have been identified at the  $\beta$ -chain loci of the inbred strains of mice (Ranney and Gluecksohn-Waelsch, 1955; Ranney *et al.*, 1960; Popp, 1962; Gilman 1972). Two of them Hbb<sup>d</sup> and Hbb<sup>P</sup> which give rise to the 'diffuse' trait contain a major ( $\beta^d$  major and  $\beta^P$  major respectively) and a minor hemoglobin component, while the third (Hbb<sup>S</sup>) has a single

( $\beta^S$ ) component (Ranney *et al.*, 1960; Morton, 1962; Gilman, 1972). It has been shown that the  $\beta^d$  major and  $\beta^d$  minor chains differ extensively from each other as well as from the  $\beta^S$  chain (Gilman, 1972). Likewise, the  $\beta^P$  major and  $\beta^P$  minor chains also show large differences between them (Gilman, 1972). A  $\beta$ -gene duplication has been suggested to explain the presence of major and minor bands in the 'diffuse' phenotype of mice (Hutton *et al.*, 1962; Gilman, 1972). Also, the presence of a recombinant gene as the product of an unequal cross-over between genes coding for  $\beta^d$  major and  $\beta^d$  minor has been suggested to account for a single hemoglobin type in a Thai mouse *Mus caroli* (Gilman, 1972). Similarly, there are at least 5 alleles at the  $\alpha$ -chain locus in mice (Popp, 1969). Thus two strains of mice C57BL/Cum and NB/RI have single but differing  $\alpha$ -chains (Popp, 1969) whereas the BALB/c strain of mouse has two  $\alpha$ -chains which are due to gene duplication at the  $\alpha$ -chain locus in this strain (Hilse and Popp, 1968). Similarly each of the two species CBA/Cum and C3H/Cum have differing sets of two  $\alpha$ -chains (Popp, 1969). The hemoglobins in the laboratory rat are equally diverse. Earlier electrophoretic studies on the hemoglobins of the laboratory rat (*Rattus norvegicus*) showed several components in unequal proportions (Stein *et al.*, 1971). It is now known that there are at least six hemoglobin components in the laboratory rat, some in small amounts (Garrick and Charlton, 1969; Garrick *et al.*, 1970; Garrick *et al.*, 1974; Garrick *et al.*, 1975; Garrick *et al.*, 1978). The earlier observation of Travnicek *et al.*, (1971) that the laboratory rat contains two alpha and three beta chains has been recently confirmed by Garrick *et al.*, (1975; 1978) who have characterised these chains and sequenced some of them. The latter authors further suggest that gene duplication at the  $\alpha$ -chain locus and a gene triplication at the  $\beta$ -chain locus are responsible for the multiplicity of hemoglobins in *Rattus norvegicus*. Our results with *Rattus rattus rufescens* likewise shows that each hemoglobin sample contains 2 types of  $\alpha$ - and 3 type of  $\beta$ -chains suggesting a probable gene duplication at the  $\alpha$ -chain locus and a gene triplication at  $\beta$ -chain locus. *Bandicota indica*, on the other hand, probably has a gene duplication at the  $\beta$ -chain locus since each of the 140 hemoglobin samples showed one  $\alpha$ -chain and two  $\beta$ -chains.

From the foregoing consideration, it is clear that murine hemoglobins provide examples of gene iteration at the hemoglobin loci. Instances of clusters of genes producing sets of closely related globin polypeptide chains are not uncommon in eutherian mammals. In fact, in five of the mammalian orders represented in figure 1 namely *Primates*, *Perissodactyla*, *Artiodactyla*, *Rodentia*, and *Carnivora* gene duplications at the hemoglobin loci have been established.

The first gene cluster to be demonstrated was a pair of linked genes coding for the  $\beta$ -and  $\delta$ -chains of humans (Ceppellini, 1959). The evolutionary origin of this duplication has now been traced to the anthropoid stem (Goodman *et al.*, 1974). However, the origin of genes coding for the embryonic zeta chains of humans and the typical eutherian  $\alpha$ -chains has been traced to an ancient duplication which occurred in the basal eutherians (Barbnabas *et al.*, 1978). This observation is further supported by the fact that, in addition to that in man (Capp, 1970), similar zeta globins have also been found in mice and rabbits (Steinheider *et al.*, 1972; Melderis *et al.*, 1974). The more recent gene duplications in the primate line of descent are those at the respective  $\alpha$ - (Hollan *et al.*, 1972) and  $\gamma$ -chain (Huisman *et al.*, 1972) gene loci of humans. The Perissodactyl branch also shows a recent gene duplication at the  $\alpha$ -

chain locus in that the two  $\alpha$  chains of horse differ by a single amino acid substitution at the 60th residue position (Kilmartin and Clegg, 1967). Similarly, duplication at the  $\alpha$ -chain loci of dog (Dresler *et al.*, 1974) may also be of recent evolutionary origin. The Artiodactyl branch, on the other hand, shows a series of duplications during its evolutionary history. Among these, the duplications in water buffalo—I $\alpha$ —II $\alpha$  (Balani and Barnabas, 1965), bison—I $\alpha$ —II $\alpha$  (Huisman, 1974), deer—I $\alpha$ —I $\alpha$  (Harris *et al.*, 1972), goat—I $\alpha$ —II $\alpha$  (Huisman *et al.*, 1980; John and Barnabas, 1975) and barbary sheep—I $\alpha$ —I $\alpha$  (Wilson *et al.*, 1970), appear to be relatively more than those in bovid—A $\beta$ -F $\beta$  (Huisman, 1974) and caprine A $\beta$ -C $\beta$  (Huisman *et al.*, 1968; Wilson *et al.*, 1970; John and Barnabas, 1978).

Our results on the peptide pattern analysis of the polypeptide chains of murine hemoglobins suggest that the two  $\alpha$ -chains of the house rat are closer to one another than either of them to the  $\alpha$ -chain of the bandicoot. Similarly, the three  $\beta$ -chains of the house rat resembled each other. The  $\beta$ -chains of the bandicoot likewise, are closer to one another than they are to the  $\beta$ -chains of the house rat. These results collectively suggest that hemoglobin gene diversities in each of the two species appeared independently. These may therefore be of recent origin.

### Acknowledgements

The authors thank Shri P. A. Mathew for the help in deriving the phylogenetic tree. This work is supported by a grant from University Grants Commission, New Delhi and Department of Science and Technology, New Delhi. One of the autors (J.N.) thanks I.C.M.R. for a fellowship.

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## **Presence of precursor ribosome in the ribosomal preparation from chloramphenicol-treated *Escherichia coli* AB301/105 (RNase III<sup>-</sup>).**

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**Abstract.** On sucrose gradient centrifugation, the ribosomal preparation from chloramphenicol-treated <sup>32</sup>P labelled *Escherichia coli* AB301/105 (RNase III<sup>-</sup>) showed the presence of a radioactive peak moving slower than the 70S ribosome; this peak disappeared on treatment with RNase III. The presence of precursor 30S RNA was shown in such preparations by affinity chromatography on a lysine-sepharose 4B column as well as polyacrylamide gel electrophoresis. Dialysis against low Mg<sup>2+</sup> concentration followed by sucrose density gradient electrophoresis. Dialysis against dissociation of 70S ribosome into its subunits, did not lead to the dissociation of the precursor ribosome. However, the dissociation took place upon treatment with RNase III. A tentative model of coupled rRNA transcription and ribosome assembly has been presented.

**Keyword.** Ribosomes; precursor; ribosomal RNA; RNase III; ribosome assembly.

### **Introduction**

It is well established that *Escherichia coli* ribosomal RNAs are transcribed as a single unit (30S RNA) which is subsequently processed by RNase III to precursors of 16S and 5S RNAs (Dunn and Studier, 1973; Nikolaev *et al.*, 1973, 1974; Ginsburg and Steitz, 1975). Duncan and Gorini (1975) showed the presence of a ribo-nucleoprotein particle (46S) in the lysate of *Escherichia coli* AB301/105 (RNaseIII<sup>-</sup>) and presented some evidence to indicate that this is composed of precursor rRNA (30S) and almost all the ribosomal proteins. Similarly Nikolaev *et al.* (1975) working with lysates of AB301/105 showed that the newly formed 30S preribosomal RNA moved in a complex with proteins as a particle having a sedimentation constant of 53S. A similar particle was also formed from purified RNA and 30S and 50S ribosomal proteins and could be cleaved by RNase III to yield 30S and 48S particles. There are several reports in the literature indicating that the defect in some proteins of one of the two subunits may be reflected in the assembly of the other subunit (Lewandowski and Brownstein, 1969; Kreider and Brownstein, 1971; Geyl *et al.*, 1977). These reports raise the question of whether the assembly of 50S and 30S ribosomes is coupled under *in vivo* conditions. The experiments of Duncan and Gorini (1975)

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have been repeated in this laboratory and further evidences are produced to show that the 46S ribonucleo-protein particle is indeed the precursor of 50S and 30S ribosomes. On the basis of the above, a speculative model regarding the coupled transcription and precursor ribosome assembly is presented.

### Materials and methods

*Escherichia coli* MRE600, a RNase I<sup>-</sup> strain was obtained from Dr. U. Maitra of Albert Einstein College of Medicine, Bronx, New York, USA. *Escherichia coli* AB301/105, a RNase I<sup>-</sup> and RNase III<sup>-</sup> strain, was a generous gift from late Luigi Gorini, Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts, USA. RNase-free DNase was the product of Worthington Biochemical Corporation, New Jersey, USA. Carrier-free H<sub>3</sub><sup>32</sup>PO<sub>4</sub> solution was procured from Bhabha Atomic Research Centre, Bombay. Lysine-sepharose 4B and chloramphenicol were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden and Sigma Chemical Company, St. Louis, Missouri, USA respectively.

*Escherichia coli* MRE600 (RNase I<sup>-</sup>) and *Escherichia coli* AB301/105 were respectively grown in synthetic medium (Datta and Burma, 1972) and enriched medium (Nikolaev *et al.*, 1975). Ribosomes were prepared from MRE600 by ultracentrifugation as described by Datta and Burma (1972). For preparation of ribosomes from the chloramphenicol-treated AB301/105 the cells were grown at 30°C to an absorbancy of 0.4 O.D. at 610 nm in half-diluted Luria broth and the cell pellet was suspended in 0.4 volume of TG medium (0.12M Tris-HCl, pH 7.4, 0.2 mM CaCl<sub>2</sub>, 0.002 mM FeCl<sub>3</sub>, 0.02 M KCl, 0.08 M NaCl, 0.02 M NH<sub>4</sub>Cl, 0.002 M MgSO<sub>4</sub>, and 1% glucose) containing chloramphenicol (0.4 mg/ml). For preparation of radioactive ribosomes, carrier-free H<sub>3</sub><sup>32</sup>PO<sub>4</sub> solution (40 µCi/ml) was added at this stage. The cell suspension was kept shaking for 90 min at 30°C and then chilled with the simultaneous addition of an equal volume of 0.5M NaCN and 0.075M potassium phosphate buffer pH 7.0. The suspension was centrifuged and the cell pellet was collected and washed with 10 mM tris-HCl buffer pH 7.0. Ribosomes were prepared by grinding the cell pellet for 90 min at 4°C with twice its (wet) weight of alumina (A-305) and small quantities of TMK1 buffer 0.02 M Tris-HCl, pH 7.4, 0.01M magnesium acetate, 0.03M KCl and 0.2mM mercaptoethanol. Alumina and cell debris were removed by centrifugation at 20,000×g for 20 min and the supernatant was treated for 7 min at 30°C with RNase-free DNase (100 µg per 10 gm of cells). The treated extract was re-centrifuged at 106,000 g for 3 h at 4°C. The sediment was resuspended in a minimum volume of TMK1 buffer.

For preparation of subunits, the ribosomal preparation was dialysed against low magnesium containing TMK2 buffer (20 mM Tris-HCl, pH 7.4, 0.3 mM magnesium acetate, 0.06M KCl and 1.4 mM mercaptoethanol) for 24 h. The density gradient centrifugation of this preparation on 15-40% sucrose containing TMK2 was carried out for 8 h at 130,000 g in the swing-out rotor of a Vac 601 ultra-centrifuge. When dissociation was not desired, the gradient contained TMK1 buffer. The fractions (0.25 ml) were collected and radioactivity was measured by Cerenkov radiation (Clausen, 1968).

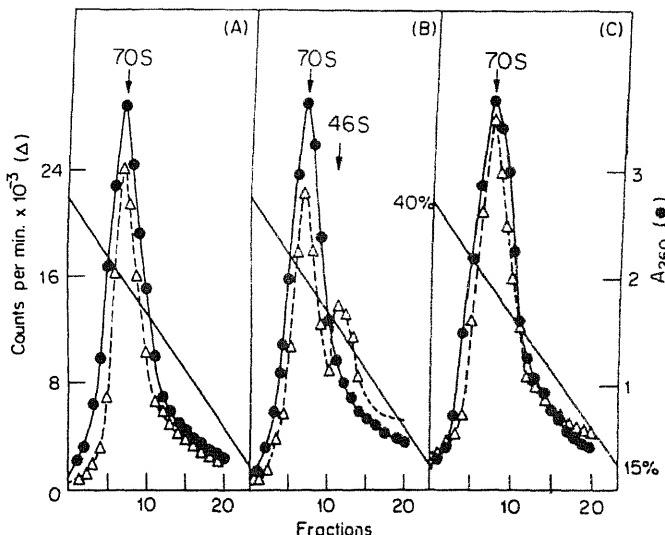
Ribosomal RNA (rRNA) was prepared by repeated phenol extraction of the ribosome followed by ethanol precipitation as described earlier (Datta and Burma,

1972). For separation of rRNAs, the affinity chromatography was carried out on a lysine Sepharose 4B column as described by Jones *et al.*, (1976). A column ( $0.8 \times 10$  cm) of lysine-Sepharose was equilibrated with 20 mM Tris-HCl, pH 7.4 containing 10 mM MgCl<sub>2</sub>. The sample of RNA (usually 20  $A_{260}$  units) was loaded on the column which was eluted with the equilibrating buffer containing NaCl of increasing concentration (a linear gradient of 0.05M to 0.5M). Fractions (1 ml) were collected and their absorbancy ( $A_{260}$ ) and radioactivity were measured.

RNase III was purified according to the method of Suryanarayana and Burma (1975) with some modification. The treatment of ribosome (25  $A_{260}$  units and  $4 \times 10^4$  counts/min) or RNA (15  $A_{260}$  units and  $3 \times 10^4$  counts/min) with 6 units of purified RNase III was done at 37°C for 30 min in a total volume of 0.25 ml containing 40 μmol glycine-KOH, pH 10, 100 μmol of KCl and 5 μmol of magnesium acetate. After incubation, the mixture was cooled to 0°C and then layered on the top of a 15–40% sucrose gradient. The subsequent procedure was the same as described before.

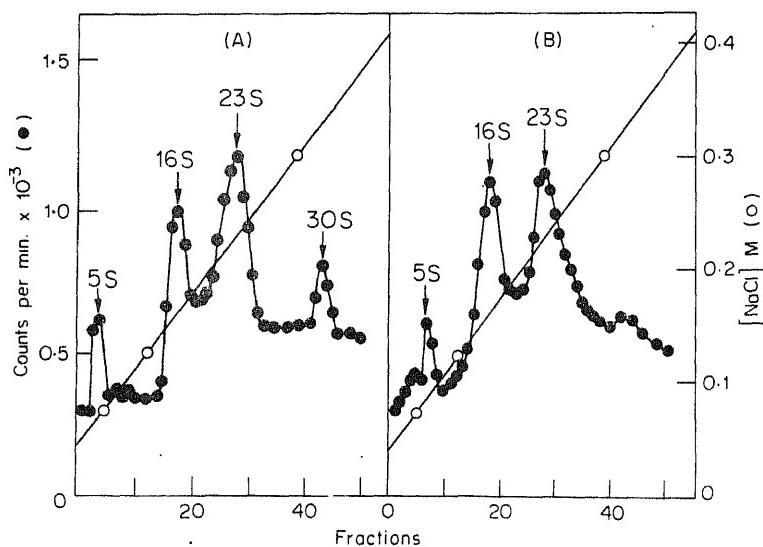
## Results

It is clear from the results presented in the figure 1A and B that the absorbancy profiles of the ribosome from *Escherichia coli* AB301/105 treated with chloramphenicol and *Escherichia coli* MRE 600 are similar showing the presence of 70S ribosomes. However, the radioactivity profile shows an extra peak sedimenting at a slower rate in the ribosomal preparation from AB301/105 (figure 1B). This peak disappears when the preparation is treated with RNase III (figure 1C). This peak seems to have a sedimentation rate between 50S and 30S and is presumably due to 46S ribosomes as detected by Duncan and Gorini (1975) in the lysate of AB301 cells.



**Figure 1.** Conversion of precursor ribosome to 70S ribosome by the action of RNase III. <sup>32</sup>P labelled ribosomes from *Escherichia coli* MRE600(A) and chloramphenicol-treated *Escherichia coli* AB 301 (B) were isolated and layered on a 15–40% sucrose gradient. The centrifugation was done as described under Materials and Methods. The ribosomal preparation from *Escherichia coli* AB301 treated with RNase III (C) was similarly run. The details have been described under Materials and Methods.

In order to demonstrate the presence of precursor rRNA in such a ribosomal preparation, rRNA was isolated by phenol extraction as described under Materials and Methods. The presence of precursor RNA was checked by two independent methods (i) polyacrylamide gel electrophoresis and (ii) affinity chromatography on a lysine-Sepharose 4B column. The results obtained with polyacrylamide gel electrophoretic method are not being presented as the standard patterns reported by others (Dunn and Studier, 1973; Nikolaeva *et al.*, 1973) were obtained with the preparation from the chloramphenicol-treated AB301 cells. Other than 23S, 16S and 5S RNAs, an extra band moving slowest of all, was present. This was absent in the preparation from MRE600 and also disappeared on treatment of the preparation from AB301 with RNase III. Affinity chromatography on lysine-Sepharose 4B column has been introduced comparatively recently as an efficient method for the separation of rRNAs (Jones *et al.*, 1976). The presence of 30S RNA in the rRNA preparation could also be demonstrated by this technique (figure 2). As expected for such a column, 30S RNA is eluted last and clearly separated from 16S and 23S RNAs (figure 2A). Treatment with RNase III leads to a considerable reduction of this peak and peaks corresponding to 10S and 23S appear (figure 2B) indicating that this might be the precursor rRNA. This 30S RNA was isolated and treated with RNase III and it was found that this gives rise to 23S, 16S and 5S RNAs confirming to identity with precursor rRNA (results not presented). The presence of precursor 30S RNA in the ribosomal preparation confirms that it is a component of the ribosome.

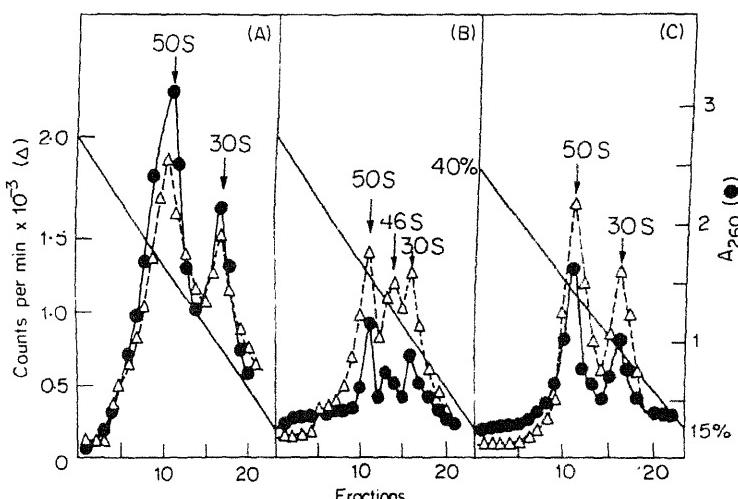


**Figure 2.** Identification of precursor rRNA present in the ribosomal preparation from the chloramphenicol-treated *Escherichia coli* AB301 cells.

Radioactive rRNAs were isolated from  $^{32}\text{P}$  labelled *Escherichia coli* AB301 ribosome and loaded on a lysine-Sepharose 4B column (A) as described under Materials and Methods. The preparation treated with RNase III was similarly chromatographed (B).

If the species of ribosomes constituted of 30S RNA exists, then these are expected not to dissociate into subunits at very low  $Mg^{2+}$  concentration, unlike 70S ribosomes. In order to verify this, ribosomes were prepared in the usual way from the chloramphenicol-treated  $^{32}P$  labelled *Escherichia coli* AB301/105 cells as described under Materials and Methods. This preparation was dialysed for 24 h against low  $Mg^{2+}$  containing buffer (dissociating buffer) in order to dissociate the subunits. As expected, the 70S ribosomal preparation from MRE600 gives rise to two peaks corresponding to 50S and 30S ribosomes (figure 3A). It is interesting to note that an extra peak (other than 50S and 30S ribosomes) is detectable under conditions causing subunit dissociation in the preparation from *Escherichia coli* AB301/105. The position of this peak is between 50S and 30S ribosomes and coincides with the precursor ribosomal peak as detected earlier (figure 1B). Thus even under dissociating conditions the precursor ribosome does not dissociate into 50S and 30S ribosome; this is to be expected if they are assembled on a single chain of RNA.

In order to verify whether the extra ribosomal peak is really the precursor of 50S and 30S ribosomes, the ribosomal preparation from chloramphenicol-treated *Escherichia coli* AB301 cells was treated with RNase III and then subjected to extensive dialysis under dissociating conditions followed by sucrose gradient centrifugation. The middle peak as obtained earlier (figure 3B) disappears and only two peaks corresponding to 50S and 30S ribosomes are obtained (figure 3C). It is quite clear that the precursor ribosome dissociated into 50S and 30S ribosomes following treatment with RNase III. This experiment strongly advocates the existence of a precursor ribosome and its conversion to 50S and 30S ribosomes.



**Figure 3.** Demonstration of the integrity of the precursor ribosome under subunit dissociating conditions.

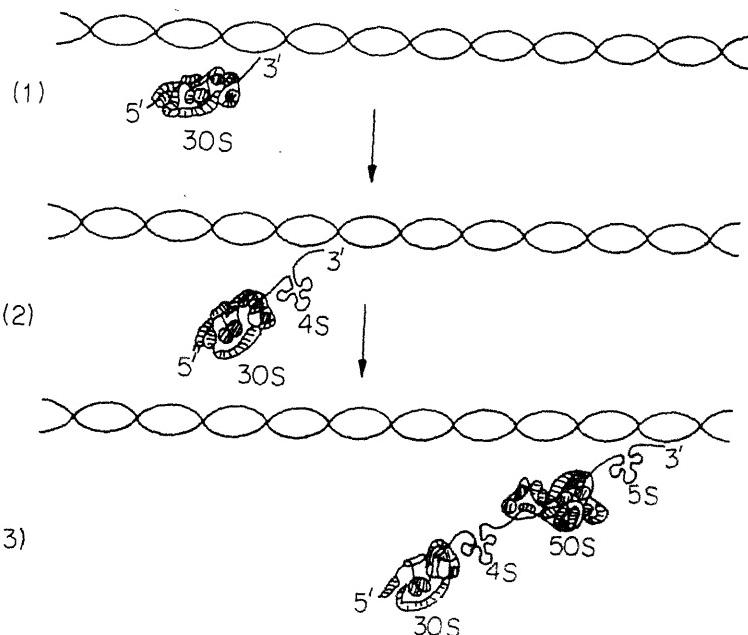
$P^{32}$ -labeled ribosomes from *Escherichia coli* MRE600 (A) as well as *Escherichia coli* AB301 (B) were separately dialysed against 0.3mM  $Mg^{2+}$  containing TMK2 buffer and loaded on 15-40% sucrose gradients. *Escherichia coli* AB301 ribosomal preparation was also treated with RNase III and then dialysed (C) as described under Materials and Methods. The remaining procedure was the same as described earlier.

## Discussion

As already mentioned, Duncan and Gorini (1975) produced evidence to indicate the presence of a ribosomal population which has a sedimentation constant of 46S and is constituted of precursor 30S RNA and almost all the ribosomal proteins, in chloramphenicol-treated *Escherichia coli* AB301/105 Nikolaev *et al.* (1975), on the other hand, showed the movement of precursor 30S RNA into a ribonucleoprotein particle with a sedimentation constant of 53S. They were also able to reconstitute the 53S particles from 30S RNA and ribosomal proteins (Nikolaev and Schlessinger, 1974). The present investigations produce further evidence to indicate the existence of precursor ribomes in the population of ribosomes isolated from the cloramphenicol-treated *Escherichia coli* AB301/105 cells. Not only does the precursor ribosome disappear on treatment with RNase III as observed by others (Duncan and Gorini, 1975; Nikolaev *et al.*, 1975) but also such a ribosomal population does not dissociate into subunits until and unless it is treated with RNase III. This is a direct demonstration of the existence of the two subunits in a linked form. Incidentally, affinity chromatography on a lysine-Sepharose 4B column has been shown here to be a very useful method like sucrose density gradient centrifugation and polyacrylamide gel electrophoresis (Ginsburg and Steitz, 1975; Schlessinger *et al.*, 1974) in the detection of precursor RNA.

It has not yet been possible to demonstrate the presence of such ribosomal populations in normal cells and therefore it may be argued that such ribosomal assembly takes place under unphysiological conditions (chloramphenicol treatment of RNase III<sup>-</sup> cells) when precursor RNA (30S) accumulates. However, the observation that the assembly of 50S ribosome is interfered with, due to the mutation of a protein in the 30S subunit is a highly corroborating fact. For example, it is known that a 30S r-protein is altered in the spectinomycin-resistant mutant spec-49, an assembly defective mutant, but in this mutant the assembly of the 50S particle is also defective (Nashimoto and Nomura, 1970). The S2 and S3 suppressor mutations suppressed the defect in 50S assembly in spec-49 (Nashimoto and Uchida, 1975). The assembly mutants with altered S4 (Lewandowski and Brownstein, 1969; Kreider and Brownstein, 1971), S8 (Geyl *et al.*, 1977) and S20 (Wittmann *et al.*, 1974; Bock *et al.*, 1974) also seemed to have defective 50S assembly. Furthermore, the cold sensitivity of the S8 mutant apparently could be suppressed by mutations in a gene for a 50S r-protein, L30 (Geyl *et al.*, 1977). These observations suggest that 50S assembly may be coupled to 30S assembly. However, there are two assembly mutants that appeared to have a defect in 30S assembly only (Rosset *et al.*, 1971; Nomura, *et al.*, 1977). Thus it is still not clear whether there is any obligate coupling of 50S and 30S assembly *in vivo* (Nomura *et al.*, 1977). If it is assumed that the ribosomal assembly starts along with the transcription of 30S RNA it is most likely that the assembly of the small subunit will precede that of 50S, 16S rRNA being located in the 5'-end and a defect in 30S subunit may be reflected in the assembly of 50S subunit which is expected to assemble later (figure 4). It is, however, difficult to explain by such assumption, the results obtained with *erythro* 8 mutant which modifies the large subunit but blocks the assembly of both subunits (Pardo and Rosset, 1977). However, *in vitro*, transcriptionally-coupled assembly of *Escherichia coli* ribosomal subunits

has been recently demonstrated (De Narvaez and Schaup, 1979). The coupling of the transcription of rRNA and the assembly of ribosomal proteins will naturally be to the advantage of the cell.



**Figure 4.** Model of coupled transcription and ribosome assembly. The details have been discussed in the text.

### Acknowledgements

The financial assistance received from the Jane Coffin Childs Memorial Fund for Medical Research, USA and the University Grants Commission, India is being gratefully acknowledged. The discussion on the work presented here with Dr. Annamaria Torriani-Gorini of the Massachusetts Institute of Technology, Cambridge, USA helped considerably in the preparation of the manuscript.

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## ACKNOWLEDGEMENTS

The editorial board wishes to place on record the valuable assistance rendered by the following scientists in reviewing manuscripts received for publication in the *Journal of Biosciences*.

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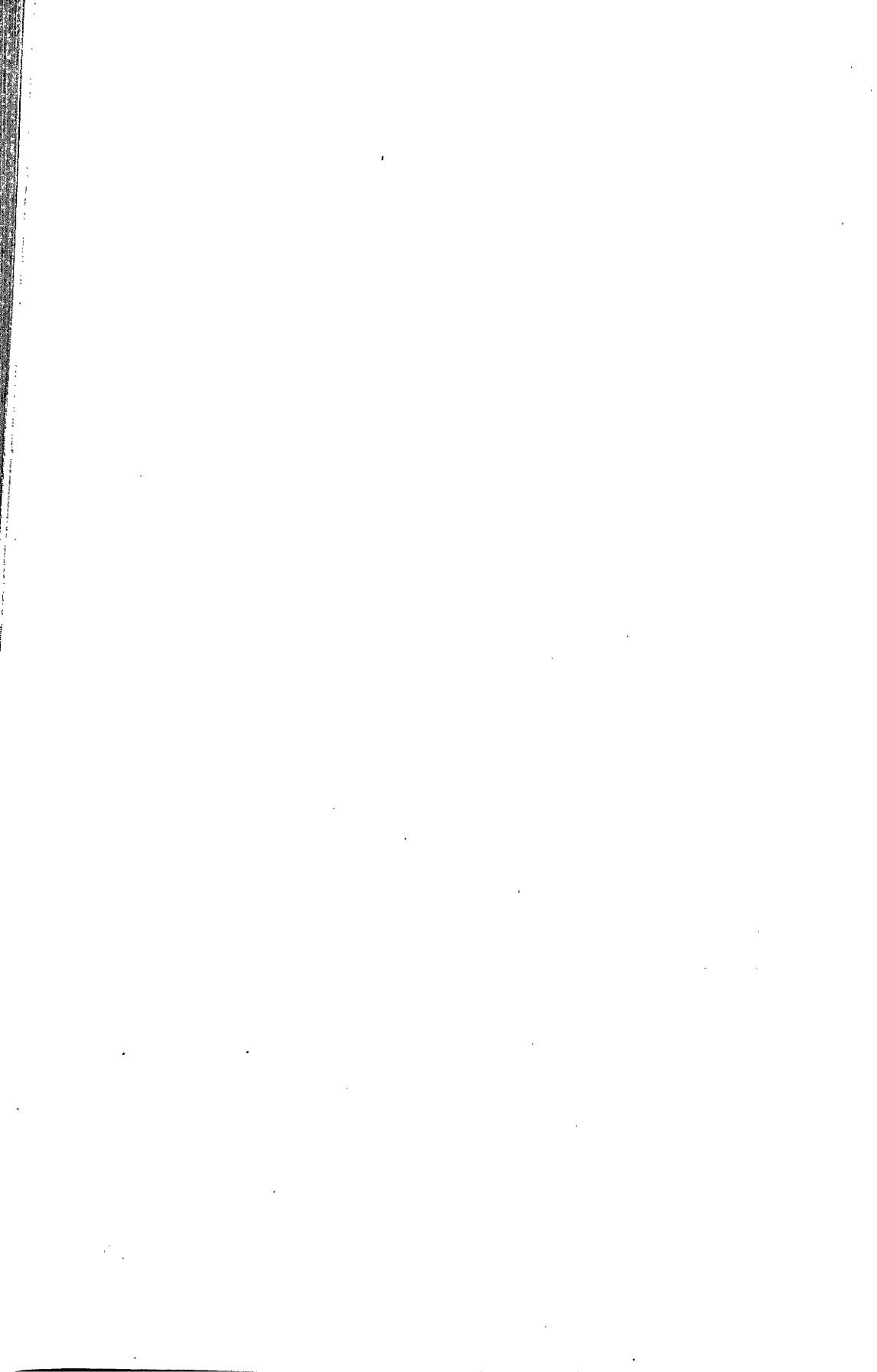
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# Journal of Biosciences

Vol. 2, January-December 1980

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